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13. ABSTRACT (Maximum 200 Words)

This is an annual report of an IDEA Award to study the molecular mechanisms driving acquired antiestrogen resistance. We have identified several genes associated with resistance to ICI 182,780 (Fulvestrant. Faslodex). Our initial studies with NFkB have now been published (*Mol Cell Biol,* 23: 6887-6900, 2003). Further functional studies on the role of other genes are in progress, and we have made good progress with IRF-1 using a dominant negative construct. We also have built and tested neural network predictors to separate several antiestrogen resistance phenotypes. We have completed and published a method that allows us to visualize very high dimensional data while maximizing the discriminant information in the molecular profiles (*J Signal Process Systems,* in press, 2003). Thus, we have made substantial progress in defining initial components of a broader antiestrogen signaling network that is directly associated with acquired antiestrogen resistance.

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2. Wang, Z., Zhang, J., Lu, J., Lee, R., Kung, S.-Y., Clarke R. & Wang Y. Discriminatory mining of gene expression microarray data. *J Signal Process Systems,* in press.

3. **Clarke, R.,** Liu, M.C., Bouker, K.B., Gu, Z., Lee, R.Y., Zhu, Y., Skaar, T.C., Gomez, B., O'Brien, K., Wang, Y., Hilakivi-Clarke, L.A. "Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling." *Oncogene,* 22: 7316-7339, 2003.

4. Welch, J.N. **& Clarke, R.** "ErbB-2 expression and drug resistance in cancer." *Signal*, 3: 4-9, 2002. (review – this was presented as being in press in the prior report).

5. Liu, Aiyi, Zhang, Ying, Gehan, Edmund **& Clarke, R**. "Block principal component analysis with application to gene microarray data classification." Statistics in Medicine, 21:3465-3474

INTRODUCTION

Antiestrogens have been successfully used in the management of breast cancer since the first clinical trial of Tamoxifen (TAM) in 1971 (3). TAM produces a significant increase in both overall and recurrence-free survival but resistance almost inevitably arises in most patients (5,6). We hypothesize that one form of acquired antiestrogen resistance reflects the altered expression of what were previously estrogen-regulated genes. We further hypothesize that only a subset of all estrogen (E2)-regulated genes, those comprising a specific gene network, is responsible for the resistance phenotype. Since TAM (triphenylethylene) and ICI 182,780 (steroidal) induce different ER conformations, we also hypothesize that the consequent patterns of gene regulation will be different and dictate the presence/absence of crossresistance among antiestrogens.

To address these hypotheses, we have generated novel E2-independent and antiestrogen resistant variants of the E2-dependent, MCF-7 human breast cancer cell line (MCF7/MIII, MCF7/LCC1, MCF7/LCC2, MCF-7/LCC9) - recently reviewed in (1). We also have assembled a panel of additional resistant cells from within this institution and from other investigators. These include additional antiestrogen resistant MCF-7 variants (LY2, R27, R3, MCF-7RR), all of which express ER, and the ER-negative ZR-75-1 (ZR75/LCC3, ZR-75-9a1) and T47D (T47Dco) variants. Other resistance models are currently being obtained from other laboratories or being generated by selection *in vivo* selection against TAM in athymic nude rats (rats and humans perceive TAM as a partial agonist, mice perceive TAM as a pure agonist).

This is an Idea Award to study the genes and patterns of genes expressed in acquired antiestrogen resistance in cell culture models. The PI will apply new, state-of-the-art technologies to identify key endocrine-regulated molecular pathways to apoptosis/proliferation. By identifying key components of these pathways, we may be able to predict response to first-line and crossover antiestrogenic therapies, and/or provide novel therapeutic strategies for antiestrogen resistant tumors.

Antiestrogen Resistance. Most breast tumors that initially respond to TAM recur and require other endocrine or cytotoxic therapies (6). Despite over 10 million patient years of experience with TAM, the precise mechanisms that confer acquired resistance are unknown (1). Absence of ER expression is clearly important for *de novo* resistance (1). ER expression is <u>not</u> lost in most breast tumors that acquire antiestrogen resistance (9). Currently, there is little compelling evidence that expression of ER splice variants and mutant ER contribute significantly to antiestrogen resistance in patients (1,10). While the importance of wild type ERα is established as a mediator/predictor of antiestrogen responsiveness, that of ERβ remains unclear. ERα may be the predominant species in most ER+ breast tumors (11,13), and is associated with a better prognosis (7). ERβ is associated with a poorer prognosis, absence of PgR, and lymph node involvement (4,13). One small study reported higher ERβ mRNA levels in resistant tumors (12). However, this association could not be separated from that between ERβ and a more aggressive phenotype (4,13). Some studies report activities independent of ER function, which may initiate events that are necessary but not sufficient for antiestrogen-induced effects (1). Our research team has recently reviewed in detail the potential mechanisms of antiestrogen resistance (2).

BODY OF REPORT

Our purpose is to evaluate a series of antiestrogen responsive and resistant breast cancer cell lines for their patterns of gene expression. We will explore these data using state-of-the-art pattern analysis and statistically-based methods that apply both statistical and information theory. We also will apply the more computationally simplistic methods used by others in the field.

In a prior report, we made one change to the specific aims and Statement of Work. Our collaborations with Dr. Wang's group at Catholic University of America (now at Virginia Tech – Dr. Wang recently moved to VA Tech's Alexandria Research Institute), in which we have begun to develop and test several new algorithms for mining the high dimensional data sets produced by gene expression microarray analyses, continue throughout this award.

Specific Aims (unchanged)

Specific Aim 1: use gene microarrays to identify differentially expressed genes in a panel of breast cancer cell lines.

Specific Aim 2: explore the data from Aim 1 to identify those differentially expressed gene clusters most closely associated with acquired antiestrogen resistance and test further novel algorithms for the analysis of gene expression microarray data.

Specific Aim 3: begin to assess the likely functional relevance of representative members of these clusters and study their expression in human breast cancer biopsies.

Long term aims: establish a pattern(s) of gene clusters that can predict antiestrogen responses in patients. This could lead to a more effective identification of candidates for specific antiestrogen therapies and identify those patients least likely to respond and who may benefit from an early initiation of cytotoxic chemotherapy.

KEY RESEARCH ACCOMPLISHMENTS

TASK 1: *Use gene microarrays to identify differentially expressed genes in a panel of breast cancer cell lines.*

We have completed this aim with the possible exception of arraying RNA against the new Affymetrix GeneChip that contains the entire human genome (released in Oct, 2003). For the cDNA arrays (Research Genetics), we have arrayed and *individually* aligned all of the digitized images. We used Pathways vs. 4.0 and independently aligned each of the ~4,000 spots/array; this is very time consuming but provides much higher quality data than using only the software to align automatically each spot. We have also generated some simple algorithms to more effectively assess bleeding effects - a problem with these radiolabeled probes where the signal from an abundant mRNA bleeds into an adjacent signal. This approach will be included in a manuscript currently in preparation and is briefly described below.

Data Preprocessing: Pathways™ 4.0 software algorithms (Research Genetics, Inc.) corrected the local nonspecific binding of the probe to filter for each spot (background correction). Intensities of both local background and spot for each gene were measured geometrically. Each gene's

average intensity was measured specifically within a circle target that is 75% of the circle enclosing the gene spot. Additionally, from 125% outside the enclosed circle to 150% outside the circle (in square form), the average local background intensity for each gene was measured.

Radioactive signal bleeding from neighboring cDNA spots is a major confounding factor in this microarray platform. Consequently, we generated a simple algorithm to detect compromised signals. For each gene on the nylon filter, bleeding effects were approximated by calculating the difference between the local and global background signals. Global background was estimated by taking the mean of the lowest 20% of all local background intensities from cDNA-free regions on the nylon filter. Genes were called as being "very low/not detected" if their raw intensity signals were within three standard deviations of the global background mean.

The difference between local and global background was calculated as a percentage of the raw intensity value of that gene. A "percentage-above" threshold was constructed to indicate that the radioactivity from neighboring spots had bled into the spot of interest, whereas a "percentage-below threshold" was used to indicate that the bleeding effects were negligible. Invalid genes were eliminated from the analyses.

Because the calculated estimate of the bleeding effect was empirically derived, a range of threshold values was inspected and statistical analyses were re-evaluated on the data sets with the varying threshold values used to assess likely radioactive bleeding. A threshold range of 21%-40% was necessary to objectively tag and eliminate affected genes by considering the bleeding effects of 1) the local spot, 2) the neighboring spots into the local background area, and 3) nonspecific local background hybridization. In addition, for genes of interest, manual spot visualization was performed to assess further the bleeding effect estimate.

TASK 2: *Explore the data from Aim 1 to identify those differentially expressed gene clusters most closely associated with acquired antiestrogen resistance.*

We have essentially competed this Task, using both the Clontech and Research Genetics platforms. Our previous report included tables of the data from the Clontech arrays. We now include a Table of genes from the Research Genetics platform. We would not expect to find the same genes since there are many genes on the ResGen filters that are not on the Clontech filters. Furthermore, since the probes are prepared and labeled differently, the signal scale for each gene is different and, therefore, the ability to identify differential expression is not identical.

LCC1 (n=3; responsive), LCC2 (n=3; resistant), LCC9 (n=3; resistant), LY2 (n=3; resistant), and RR (n=3; resistant) are estrogen independent MCF-7 breast cancer cell line variants that are either resistant or responsive to known Selective Estrogen Receptor Modulators. These cell lines were arrayed against Research Genetics GF211 (NamedGenes™) nylon filters probing radiolabeled-^{33}P cDNA targets. A Molecular Dynamics Storm Phosphorimager was used to scan the radiolabeled filters, and Resgen Pathways™ 4.0 imaging software measured the densitometric readings from the digital scans.

In-depth analyses/mining were done on Mathworks MATLAB™ with established and developing algorithms. After excluding low threshold signals (<0.1 in each group) and bleeding effects contributed by the neighboring spots (as described above), we initially filtered the 4,324 dimensional array to 1,882 genes. At the top level, we globally visualized the 1,882-dimensional data from the breast cancer variants using Principal Component Analyses (PCA). A nonlinear separation is seen in this projection between the antiestrogen resistant and responsive groups, suggesting that phenotype separation is possible and more samples are needed to clearly define the boundary between the two antiestrogen resistant/responsive breast cell models (this is data

visualization, not data analysis) in this full dimensional data space.

Dimensionality reduction by gene selection was necessary to identify an accurate and robust discriminant gene expression profile. We used a novel profile selection algorithm that filters the 1882-D data set by the highest signal-to-noise ratios, and then eliminates genes by both assessing their contribution to the profile's strength and by maximizing the trace of a weighted Fisher's scatter matrix. We applied the algorithm to the two different antiestrogen resistant/responsive phenotypes from the MCF-7 variants using either all samples or random multiple subsets of samples. From using all the of estrogen independent MCF-7 variant cell lines, the algorithm identified a 20 gene subset that discriminates accurately between the two phenotypes. This 20-dimensional data set was projected into 3-dimensional space with both PCA and our new Discriminant Component Analysis (DCA) (14). The antiestrogen resistant/responsive phenotypes are now linearly separable in both visualizations (see Figure below).

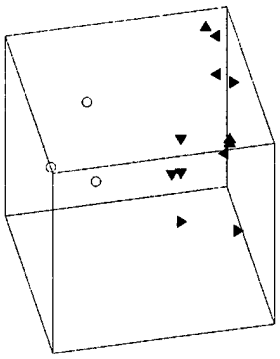
We iteratively tested a neural network Multi-Layer Perceptron composed of three hidden nodes in one hidden layer. Four random samples from the antiestrogen resistant samples were chosen as independent data sets, and the remaining samples were used to train a neural network to predict the class of each independent sample (trained using the 20 genes selected above). After 100 cycles, the MLP predicted the independent samples with an overall accuracy of 92.5%, as a proof of principle that antiestrogen resistance can be adequately predicted.

UNIGENE ID	Gene Description	RESPONSIVE / RESISTANT	RESISTANT / RESPONSIVE	Student t-test (equal)
Hs.77858	mesenchyme homeo box 2 (growth arrest-specific homeo box)	2	0.50	5.06037E-05
Hs.77515	inositol 1,4,5-triphosphate receptor, type 3	2	0.50	0.000504835
Hs.404	myeloid/lymphoid or mixed-lineage leukemia (trithorax (Drosophila) homolog); translocated to, 3	2	0.50	0.000328254
Hs.151777	eukaryotic translation initiation factor 2, subunit 1 (alpha, 35kD)	4	0.25	0.000748487
Hs.271980	mitogen-activated protein kinase 6	2	0.50	0.000633155
Hs.172210	MUF1 protein	6	0.20	0.000361767
Hs.1420	fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)	2	0.50	0.000383285
Hs.26014	activin A receptor, type II	3	0.33	1.02059E-05
Hs.349092	ESTs, Weakly similar to I38022 hypothetical protein [H.sapiens]	4	0.25	6.81344E-05
Hs.75447	ralA binding protein 1	4	0.25	0.000316546
Hs.74294	aldehyde dehydrogenase 7 family, member A1	2	0.50	0.000100641
Hs.82002	endothelin receptor type B	3	0.33	0.000569089
Hs.4082	lectin, galactoside-binding, soluble, 8 (galectin 8)	5	0.20	1.83147E-05
Hs.4187	hypothetical protein 24636	5	0.20	4.789E-07
Hs.75564	CD151 antigen	3	0.33	0.000340471
Hs.77613	ataxia telangiectasia and Rad3 related	2	0.50	0.000390206
Hs.74101	spleen tyrosine kinase	2	0.50	0.000482157
Hs.89691	UDP glycosyltransferase 2 family, polypeptide B4	2	0.50	0.001938969
Hs.48876	farnesyl-diphosphate farnesyltransferase 1	2	0.50	0.00018038
Hs.98074	itchy (mouse homolog) E3 ubiquitin protein ligase	3	0.33	0.000154619

These observations strongly implicate the 20 genes as being both differentially expressed and discriminatory between antiestrogen sensitive and resistant cells. The data also suggest that some of these genes may be functionally involved in conferring acquired antiestrogen resistance in breast cancer cells. Further analysis of these genes and the entire data set continue as we apply additional methods to mine the data.

In our last report we included our new normalization publication (*IEEE Trans Inf Technol Biomed,* 6: 29-37, 2002). We now include a publication on our novel "block principal components analysis" method for exploring gene expression microarray data. A reprint is included in the appendix.

We have also derived a new method for multidimensional scaling called discriminant components analysis. Rather than focusing on capturing data variance, as is done using principal component analysis, this new method maximizes the discriminating components in the data. The manuscript has been accepted for publication in the informatics literature (*Journal of Signal Processing Systems*) and a preprint is included in the appendix.



DCA projection of 20-D data set showing linear separability of antiestrogen sensitive and resistant profiles. o=LCC1 (sensitive; n=3); ▲=LY2 (n=3; resistant);▼=LCC9 (n=3; resistant);◀=LCC2 (n=3; resistant); ▶=RR (n=3; resistant); Total variance covered by top 3PC: 75%

TASK 3: *Begin to assess the likely functional relevance of representative members of these clusters and study their expression in human breast cancer biopsies.*

To maintain focus within this application, we have limited our initial studies to NFkB and IRF-1. Our intention is to obtain sufficient preliminary data to support an R01 or DOD application focused on these two genes and their interactions in antiestrogen resistance. We have continued to study the role of our dominant negative interferon regulatory factor-1 (dnIRF-1). We have now made excellent progress on this aspect of the study and have submitted a manuscript showing the ability of dnIRF-1 to block the proapoptotic effects of ICI 182,780 but not the cell cycle arrest effects of the antiestrogen. Our ability to separate these two components of sensitivity to antiestrogens has several important implications. For example, selectively increasing the proapoptotic effects of antiestrogens may be an effective means to improve their ability to increase overall survival in patients because this should increase the proportion of cells undergoing apoptotic cell death. Cells that are only growth arrested may survive and thereby have more opportunities to adapt, acquire resistance, and generate subsequent disease recurrence. A copy of the manuscript will be included with the final report.

We have also made good progress in our initial studies on NFkB. In a collaboration with Dr. Christine Pratt at the University of Manitoba, we now implicate NFkB in estrogen-independence. These data are consistent with the increased sensitivity of our antiestrogen resistant cells to the natural inhibitor of NFkB (parthenolide). Our data with parthenolide were included in the paper by Gu *et al.* that we included with last years report (8). The study with Dr. Pratt's laboratory is included with this report (see appendix).

Key Research Accomplishments (bulleted)

- Completed and published manuscript describing data implicating NFκB in estrogen independence.
- Completed microarray data analysis of cell lines.
- Built an accurate neural predictor of antiestrogen responsiveness based on the microarray data collected above.
- Completed and published (in press) a new algorithm for microarray data visualization based upon maximizing the discriminant data among experimental groups.
- Completed and submitted our initial studies of the role of IRF-1 in ICI 182,780 mediated cell signaling.

Reportable Outcomes

Reportable outcomes are presented as manuscripts and abstracts.

Manuscripts and Abstracts

We have published several studies directly related to the funded work, including a major review in the journal *Oncogene.*

Manuscripts

1. Pratt, M.A.C., Bishop, T.E., White, D., Yasvinski, G., Ménard, M., Niu, M.Y. **& Clarke, R.** "Estrogen withdrawal-induced NF-κB and Bcl-3 expression in breast cancer cells: roles in growth and hormone independence. " *Mol Cell Biol,* 23: 6887-6900, 2003.

2. Wang, Z., Zhang, J., Lu, J., Lee, R., Kung, S.-Y., Clarke R. & Wang Y. Discriminatory mining of gene expression microarray data. *J Signal Process Systems,* in press.

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4. Welch, J.N. **& Clarke, R.** "ErbB-2 expression and drug resistance in cancer." *Signal*, 3: 4-9, 2002. (review – this was presented as being in press in the prior report).

Reprints of papers #1-3 and a preprint of #4 are included in the appendix.

Abstracts

1. Zwart, A., Lee, R.Y., Zhang, J., Wang, J., Wang, Y. **& Clarke, R.** "mRNA profiles from MCF-7 variants are used to predict antiestrogen resistance/responsive phenotypes." *Proc 85th Annual Meeting Endocrine Society* 146, 2003.

Conclusions

We have made excellent progress in our studies on the molecular characterization of antiestrogen resistance, which is evident in our productivity as measured by publications and new preliminary data. The study is on-track and the amount of data accumulating is considerable. Several new algorithms under development are showing good performance in our initial analyses. Our data with NFκB, IRF-1, and the dnIRF-1 are encouraging and suggest we are on the right track to identifying new signal transduction pathways associated with acquired antiestrogen resistance. For example, these data show that resistant cells are more sensitive to inhibition of NFκB. Overexpression of IRF-1, which is suppressed by estrogens and induced by antiestrogens, is associated with reduced cell proliferation, and the dnIRF-1 data indicate that ICI 182,780 signals to apoptosis primarily through IRF-1.

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Estrogen Withdrawal-Induced NF-κB Activity and Bcl-3 Expression in Breast Cancer Cells: Roles in Growth and Hormone Independence

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About one-third of breast cancers express a functional estrogen (β-estradiol [E2]) receptor (ER) and are initially dependent on E2 for growth and survival but eventually progress to hormone independence. We show here that ER$^+$, E2-independent MCF-7/LCC1 cells derived from E2-dependent MCF-7 cells contain elevated basal NF-κB activity and elevated expression of the transcriptional coactivator Bcl-3 compared with the parental MCF-7 line. LCC1 NF-κB activity consists primarily of p50 dimers, although low levels of a p65/p50 complex are also present. The ER$^-$ breast cancer cell lines harbor abundant levels of both NF-κB complexes. In contrast, nuclear extracts from MCF-7 cells contain a significantly lower level of p50 and p65 than do LCC1 cells. Estrogen withdrawal increases both NF-κB DNA binding activity and expression of Bcl-3 in MCF-7 and LCC1 cells in vitro and in vivo. Tumors derived from MCF-7 cells ectopically expressing Bcl-3 remain E2 dependent but display a markedly higher tumor establishment and growth rate compared to controls. Expression of a stable form of IκBα in LCC1 cells severely reduced nuclear expression of p65 and the p65/p50 DNA binding heterodimer. Whereas LCC1 tumors in nude mice were stable or grew, LCC1(IκBα) tumors regressed after E2 withdrawal. Thus, both p50/Bcl-3- and p65/p50-associated NF-κB activities are activated early in progression and serve differential roles in growth and hormone independence, respectively. We propose that E2 withdrawal may initiate selection for hormone independence in breast cancer cells by activation of NF-κB and Bcl-3, which could then supplant E2 by providing both survival and growth signals.

About 60% of all diagnosed breast cancers express estrogen receptors (ERs), and about half of these are dependent on estrogen for growth and are initially responsive to endocrine therapy (15, 25, 48). These tumors eventually acquire resistance to hormonal manipulation as part of their progression toward a more malignant phenotype, and in many instances they cease to express ERs or express mutant forms of the ER (33, 34). The MCF-7 line is a widely used prototype for estrogen-dependent breast cancer. These cells form tumors in nude mice in the presence of circulating β-estradiol (E2), and the tumors regress rapidly through an apoptotic mechanism (21) when the source of E2 is removed (29, 44). In order to study the progression of breast cancer toward a hormone-independent phenotype, sublines derived from MCF-7 cells cultured in vivo and in vitro in the presence of subphysiological concentrations of estrogen have been isolated (13, 14). MCF-7/MIII cells were isolated from a small, slowly proliferating MCF-7 tumor that arose in an ovariectomized athymic mouse, and a second passage produced MCF-7/LCC1 cells, which form E2-independent tumors with a significantly reduced latency. Both cell lines retain the parental MCF-7 level of expression of the ER but display increased expression of some estrogen-regulated genes with a concomitant loss of E2 responsiveness in vitro. Although LCC1 cells can efficiently generate tumors in nude mice in the absence of estrogen, they grow more rapidly when estrogen is present and therefore retain a degree of estrogen responsiveness in vivo (9).

The transcription factor NF-κB is composed of a heterodimer of members of the Rel family of transcription factors, including p50 (NF-κB1), p65(RelA), c-Rel, RelB, and p52(NF-κB2). Transactivation domains are absent in p50 and p52, and thus they are active only as heterodimers with other members. This family of proteins contains Rel homology domains which mediate DNA binding, dimerization, and nuclear localization. Activation of NF-κB occurs following a wide variety of stimuli, including exposure to some cytokines and several kinds of stress. Inactive NF-κB is maintained in the cytoplasm as a result of interaction with an inhibitory subunit, IκB (4), of which there are four subtypes, α,β, γ, and ε (31). NF-κB activation follows phosphorylation of IκB by IκB kinases (α or β), which in turn are activated by an NF-κB-inducing kinase called NIK (24, 38). IκB phosphorylation results in its degradation and subsequent release, allowing NF-κB translocation to the nucleus, where it regulates a large number of genes involved in inflammation, immunity, cell adhesion, and apoptosis-regulatory molecules (2). Another member of the IκB family is the oncoprotein Bcl-3, which can disrupt the association between transcriptionally inactive p50 and p52 homodimers, allowing association of a transactivating partner. Bcl-3 can also directly activate transcriptional function in these complexes (reference 31 and references therein). Much of the information regarding the role of NF-κB in cell survival has come from the study of tumor necrosis factor alpha signaling in tumor cells. While the tumor necrosis factor alpha receptor activates a caspase cascade leading to apoptosis, in most cells a concomitant activa-

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tion of NF-κB prevents cell death (3, 5). These observations led to the discovery that NF-κB regulates the activity of several survival genes, including genes for Bcl-x and inhibitors of apoptosis (IAPs) (reviewed in reference 42).

There is abundant evidence that NF-κB can promote tumorigenesis (32, 46). One of the earliest reports showed that antisense downregulation of the p65 subunit of NF-κB in fibrosarcoma cells could both inhibit tumorigenicity and cause tumor regression (22). More recently, inhibition of NF-κB activity by stable expression of a dominant negative inhibitory IκB kinase in mouse mammary tumor cells reduced their tumorigenic potential (7). Resistance to chemotherapeutic drugs is also impaired by NF-κB inhibition in tumor cells (32, 54). Studies have shown that breast cancer cell lines expressing the ER contain low levels of NF-κB DNA binding activity, while ER^- breast cancer cells display constitutively high levels of NF-κB DNA binding and correspondingly high NF-κB transactivational activity (36, 50). NF-κB activity is also induced in rat mammary glands after treatment with carcinogens and appears to increase prior to malignant transformation of mammary epithelial cells (28).

In this study we have used the ER-positive, hormone-independent LCC1 and MCF-7 parental cells to determine if and at which stage during serial breast cancer cell progression toward hormone independence these cells begin to acquire elevated NF-κB activity. Using a tumor xenograft model, we show that (i) expression of the Bcl-3 protein in MCF-7 cells augments tumor establishment and growth but is insufficient to confer E2-independence and (ii) inhibition of p65-associated NF-κB activity with a dominant form of the NF-κB inhibitor IκBα reverts the E2-independent phenotype of LCC1 cells.

MATERIALS AND METHODS

Plasmids and antibodies. Anti-p65 (A), anti-p50 (H-119), anti-p52 (K-27), anti-c-Rel (N), anti-Bax (N-20), and anti-Bcl-3 were obtained from Santa Cruz Biotechnology. Anti-Bcl-2 and anti-Bcl-xL were gifts from John Reed, La Jolla, Calif. Anti-FLAG M2 monoclonal antibody and anti-α-actin polyclonal antibody were purchased from Sigma. The 3X-NF-κB_3–luciferase construct containing three copies of the NF-κB response element from the major histocompatibility complex class I gene and a mutated version of this element (19), pRC/CMV-FLAG-tagged IκBα S32A/S36A, the superrepressor form of IκBα ($I\kappa B\alpha^{SR}$) (27), and CMV-2-FLAG-Bcl-3 were provided by A. S. Baldwin. Initial studies utilized the major histocompatibility complex reporter gene, and the κ light-chain reporter gene was obtained later since it produced higher overall enzyme values after transfection.

Cell culture and transfection. MCF-7 cells were derived from several isolates. MCF-7(early) and MCF-7(late) cells are uncloned isolates of MCF-7 at controlled passages of 46 to 48 and 157 to 159, respectively. MCF-7/MIII and MCF-7/LCC1 are ER^+, E2-independent cell lines. MIII cells were isolated from a slow-growing tumor resulting from inoculation of parental MCF-7 cells into an ovariectomized nude mouse. MIII cells were further passaged in ovariectomized nude mice and then reestablished in vitro as the continuous line MCF-7/LCC1, which forms tumors in ovariectomized mice with reduced latency (9). Both sublines were passaged fewer than 30 times after isolation. Other MCF-7 cells, not designated early or late passage, were obtained originally from L. Murphy (Winnipeg, Canada) and are of undetermined passage. MDA-MB-231 (ER^-), MDA-MB-468 (ER^-) and T47-D (ER^+) cells were obtained from the American Type Culture Collection (Manassas, Va.). The tumorigenic characteristics of many of these breast cancer cell lines have been documented (45, 49). MCF-7(40F) is an MCF-7 derivative selected for resistance to adriamycin that is E2 independent and ER^- (20). All MCF-7 derived cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL) containing a high glucose concentration, 5% fetal bovine serum (GIBCO-BRL), and 2 μg of gentamicin sulfate per ml. T47-D, MDA-MB-468, and MDA-MB-231 cells were maintained in DMEM containing 5% serum and a low glucose concentration. SKBR-3 cells were maintained in McCoy's 5A medium with 10% fetal bovine serum. Incubation was at 37°C in a 5% CO_2 humidified environment. In experiments requiring E2 depletion, cells were precultured for 7 days with several changes of phenol red-free DMEM containing 5% steroid-free fetal bovine serum that had been adsorbed to dextran-coated charcoal for 45 min at 45°C. E2 was added from a 1 mM stock in ethanol to a final concentration of 10^{-8} M for the indicated times. Transfections were performed with Lipofectamine according to the directions of the manufacturer (GIBCO-BRL). For stable transfection, pcDNA3-$I\kappa B\alpha^{SR}$-FLAG or CMV-FLAG-Bcl-3 was introduced into MCF-7/LCC1 cells, and clones were selected in medium containing 50 μg of G418 per ml as previously described (52). Resistant clones were picked and expanded, and then lysates were subjected to immunoblot analysis with the anti-FLAG antibody. For transient cotransfections, expression-reporter constructs and pcDNA3-LacZ were introduced by using Superfect or Lipofectamine according to the manufacturer's directions. Cell extracts were harvested 48 h later and analyzed in a BioOrbit 1250 luminometer by using luciferase assay reagent (Promega). Reported values represent means ± standard errors (SE) from duplicate or triplicate experiments, normalized to LacZ activity determined by methylumbelliferyl-β-glucuronide assay (1), and are representative of those from at least three separate experiments.

EMSA analysis. Electrophoretic mobility shift assays (EMSA) were performed with nuclear extracts from cultured cells or from tumors isolated as described by Osborn et al. (41). NF-κB site oligonucleotides were obtained from Promega (E3291) and end labeled with T4 polynucleotide kinase by using [γ-^{32}P]ATP (Amersham). Five micrograms of nuclear extract was mixed with 5 μl of DNA binding buffer (20 mM HEPES [pH 7.9], 0.2 mM EDTA, 0.2 mM EGTA, and 2 mM dithiothreitol in 50% glycerol), 5 μg of poly(dI-dC), and 0.2 ng of labeled probe in a final volume of 20 μl and then incubated at room temperature for 25 min. Specific bands were verified with a 10 to 125 M excess of cold oligonucleotide 10 min prior to addition of the labeled probe, and equivalence of extract loading was demonstrated by EMSA with a DNA fragment containing the consensus Sp1 binding site (Promega). Samples were loaded on a 5% native polyacrylamide gel and run in nondenaturing Tris-glycine buffer. For supershift experiments, 2 μg of each antibody was added to extracts and left for 1 h prior to addition of the labeled probe.

Tumors in nude mice. Six-week-old ovariectomized nude mice (nu/nu CD-1) were implanted subcutaneously with an estrogen release pellet (60-day-release pellet containing 0.72 mg of E2; Innovative Research of America, Sarasota, Fla.). Two days later, 2×10^6 cells derived from exponential cultures of wild-type MCF-7 cells were injected subcutaneously into the flank of the animal. For LCC1 experiments, three pooled clones of either LCC1($I\kappa B\alpha^{SR}$) or LCC1(pcDNA3) were injected subcutaneously into the contralateral flanks of 12 animals. Similarly, cell suspensions containing three pooled MCF-7(FLAG) or MCF-7(FLAG-Bcl-3) clones were injected subcutaneously into the contralateral flanks of 12 mice, while another 3 mice received only MCF-7(FLAG-Bcl-3) in one flank only. Tumors were allowed to form over a period of 4 to 6 weeks, and volumes were determined by caliper measurements as previously described (44). The E2 release pellet was then removed, and regression was monitored until the tumor reached 50% of its volume at pellet removal or over a time course as indicated, at which point the animal was sacrificed and the tumor was removed. Tumor protein lysates were prepared by snap freezing followed by pulverization under liquid N_2. After the addition of radioimmunoprecipitation assay buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 1% NP-40, 10 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of aprotinin per ml, and 0.02% sodium azide), samples were sonicated and then incubated for 30 min on ice before centrifugation at 16,000 × *g* to remove insoluble material. Protein was measured with Bio-Rad reagent.

Immunoblot analysis. Cell monolayers were washed twice with phosphate-buffered saline and lysed in 400 μl of RIPA buffer per 10^7 cells for 30 min on ice. Insoluble material was removed following centrifugation at 12,000 × *g* for 15 min, and soluble protein concentrations were determined with a Bio-Rad kit. Proteins (20 μg) were separated on SDS–7.5 or 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After exposure to primary antibody, proteins were detected with peroxidase-conjugated second antibody (Sigma) and chemiluminescent substrate (Dupont, NEN).

Immunocytochemistry and ISEL. Seven-micrometer frozen sections were cut from LCC1(pcDNA3) and LCC1($I\kappa B\alpha^{SR}$) tumors. For Bax immunostaining, sections were fixed in formaldehyde for 30 min and incubated with polyclonal Bax NH_2 terminus antibody followed by CY3-conjugated goat anti-rabbit immunoglobulin G (Jackson Laboratories). In situ end labeling (ISEL) was performed with terminal transferase and biotin-16-dUTP (Boehringer Mannheim) followed by CY2-labeled streptavidin (Amersham) as described previously (44). Sections were visualized and imaged with a Zeiss Axiophot fluorescence microscope

TABLE 1. Cell lines

Cell line	Description[a]
MCF-7 (early or late)	Human mammary adenocarcinoma; well differentiated, ER positive, $p53^{+/+}$ (39), controlled passages (early, 46 to 48 passages; late, 156 to 159 passages)
T47-D	Human mammary ductal carcinoma, differentiated epithelial, ER positive, $p53^{-/+}$ (39)
MIII	MCF-7 (controlled passage) inoculated into ovariectomized nude mice; slow-growing tumor isolated 6 months after inoculation and reestablished in vitro, ER-positive and E2-responsive in vivo (14)
LCC1	Isolated from rapidly growing tumors derived from second inoculation of MIII cells into ovariectomized nude mice and then reestablished in vitro; ER-positive and E2-responsive in vivo, increased constitutive expression of some E2-regulated genes (9)
MDA-MB-231	Human mammary adenocarcinoma; poorly differentiated, ER negative, $p53^{-/-}$ (39)
MCF-7^{ADR}(40F)	MCF-7 cells selected for 40-fold resistance to doxorubicin (adriamycin) [wild-type MCF-7 ED_{50}, 14.5 nM; MCF-7^{ADR}(40F) ED_{50}, 474 nM (20)], $p53^{mut}$ (39), ER negative

[a] ED_{50}, 50% effective dose.

equipped with Northern Eclipse software (EMPIX Imaging Inc., Mississauga, Ontario, Canada).

RESULTS

LCC1 cells display elevated NF-κB DNA binding activity. Evidence shows that ER expression in breast cancer cell lines is associated with low baseline NF-κB activity, while breast cancer cell lines devoid of ER have high constitutive levels of NF-κB activity (36). Although LCC1 cells are E2 independent they express functional ER as determined by their increased growth rate in the presence of E2 (14). Table 1 presents a summary of the cell lines and isolates used in this study. In order to determine whether NF-κB activity correlates with E2 dependence, nuclear extracts from ER^+ T47-D and ER^+ LCC1 cells were assayed for NF-κB DNA binding, and the NF-κB activity was compared with that in MCF-7 cells selected for resistance to adriamycin [MCF-7^{ADR}(40F) cells]. These cells have lost expression of the ER and are able to form tumors efficiently in ovariectomized nude mice (20). Figure 1A shows the results of an EMSA which demonstrates that, as predicted from the literature on NF-κB levels in ER^- breast cancer cell lines, MCF-7(40F) cells also contain high levels of constitutive NF-κB DNA binding activity associated with fast- and slow-migrating complexes. Conversely, T47-D cells contained very low levels of NF-κB DNA binding activity, as previously reported (36). In contrast, LCC1 cells displayed intermediate levels of NF-κB activity associated primarily with the faster-migrating complex. In order to directly compare NF-κB DNA binding activities of cells derived from the same isolate of MCF-7 cells, we prepared nuclear extracts from the early passage of parental MCF-7 cells, MIII cells, and LCC1 cells and contrasted these NF-κB DNA binding complexes with those from ER^- MDA-MB-231 cells. The results in Fig. 1B show again that LCC1 cells had high levels of NF-κB activity compared with the other MCF-7-derived cells. Similar to MCF-7(40F) cells, MDA-MB-231 cells contain a high level of constitutive NF-κB activity associated with two different complexes. The EMSA in Fig. 1C, in which an Sp1 binding site DNA fragment was used as a probe, contained equivalent amounts of the indicated nuclear extracts used for Fig. 1A and B and demonstrates that quantitative differences were not a function of extract loading. To determine the composition of the NF-κB complexes in LCC1 cells relative to ER^- cells, we supershifted nuclear extracts from LCC1 and the ER^- MCF-7(40F) and MDA-MB-231 cells with antibodies against NF-κB proteins. Figure 1D shows clearly that while p50 was present in complexes from all three cell lines, the ER^- lines contained markedly higher levels of the slower-migrating p65/RelA complex. The p50 antibody also supershifted the p65-associated complex, thus indicating that the upper complex is a heterodimer of p50 and p65. Thus, NF-κB subunits appear to be differentially activated in breast cancer cell lines. Despite its commercial designation for supershift analysis, we were unable to supershift any of the complexes with this p52 antibody or with an antibody obtained from the laboratory of A. S. Baldwin which reportedly was capable of supershifting p52 but only on an erratic basis, and therefore we cannot formally rule out that the lower-migrating complex also contains heterodimers of p52/p50. Note that all of the cell lines were cultured with identical serum concentrations, thereby eliminating the influence of differential contributions of serum growth factors on NF-κB activity.

Not all DNA-bound NF-κB is transcriptionally active (56); therefore, NF-κB transactivational activity was tested by transient transfection of an NF-κB reporter gene. The graph in Fig. 1E shows that the basal level of NF-κB–luciferase reporter gene activity in LCC1 cells was about fivefold higher than that in the E2-dependent MCF-7(early) cells. Since our supershift analysis revealed major differences between the predominant NF-κB DNA binding complexes in ER^+ cells and ER^- cells, we performed immunoblot analysis on nuclear extracts from these cell lines to ascertain the relative levels of these proteins. The results in Fig. 1F indicate that MCF-7(early) nuclear extracts contain only trace levels of p65, while LCC1 nuclei contain a slightly higher level. In contrast, the ER^-, E2-independent MCF-7(40F) and MDA-MB-231 cells harbor much higher nuclear levels of this protein. When the same extracts were assayed for p50 immunoreactivity, significantly less p50 was present in MCF-7(early) cells than in the other cells. The LCC1 and the ER^- cell lines contained similar nuclear levels of p50. Given that p50 heterodimerizes with p65 in these cells, the nuclear level of p50 would affect both the fast- and slower-migrating NF-κB complexes. The relative nuclear content of

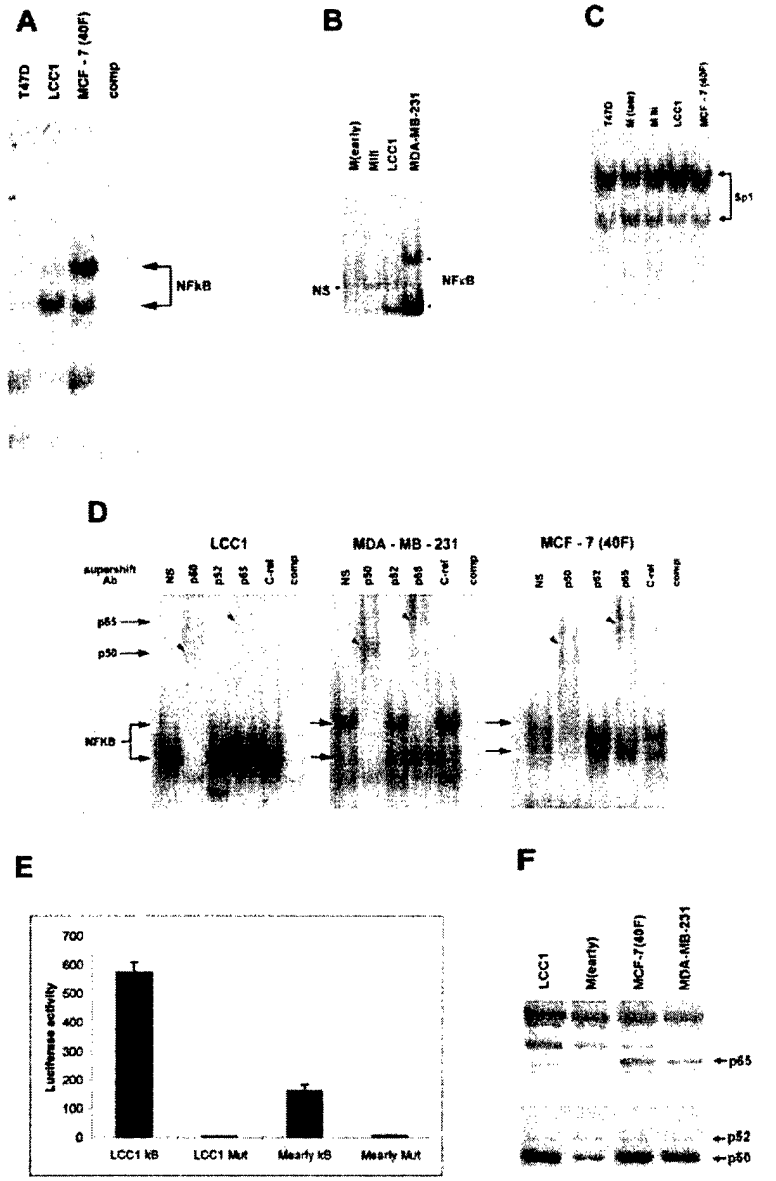

FIG. 1. NF-κB DNA binding activity increases with acquisition of E2 independence. (A and B NF-κB complexes in 5 μg of nuclear extract from T47-D, LCC1, and MCF-7(40F) cells (A) and from MCF-7(early passage), MIII, LCC1, and MDA-MB-231 cells (B) were subjected to EMSA as described in Materials and Methods, using an oligonucleotide containing a consensus NF-κB binding site. Arrows indicate fast- and slow-migrating complexes. A 10-fold excess of cold oligonucleotide (comp) was used for competition of specific bands in duplicate MCF-7(40F) and MDA-MB-231 lanes. (C) Five micrograms of the same nuclear extracts used for panels A and B was subjected to EMSA with the consensus Sp1 enhancer element as a probe. (D) Antibody supershift analysis of NF-κB complexes. NF-κB complexes from the indicated cell lines were incubated with antibodies against NF-κB proteins prior to DNA binding as described in Materials and Methods. Arrows indicate fast- and slow-migrating complexes; arrowheads identify antibody-shifted complexes. NS, normal serum; comp, a 10-fold excess of unlabeled probe was used to compete with specific bands. (E) Results from transient transfection of MCF-7(early) and LCC1 cells with the 3X-NF-κB_3–luciferase and mutant-luciferase reporter constructs. The results represent values from triplicate experiments ± SE and were normalized to β-galactosidase activity from a cotransfected pCMV-LacZ plasmid. (F) Immunoblot analysis of nuclear p65, p50, and p52 in breast cancer cell lines. Ten micrograms of each nuclear extract was reacted with either anti-p65 antibody or an anti-p50 antibody which also cross-reacts with p52.

p52 detected by this antibody was low and was essentially equivalent across all cell lines. Thus, nuclear NF-κB proteins and NF-κB DNA binding complexes are both qualitatively and quantitatively different in breast cancer cell lines and isolates grown in vitro under the same culture conditions. Progression appears to correlate with increased levels of NF-κB consisting predominantly of p50 dimers as well as with a discernible increase in the formation of p65/p50 DNA binding complexes.

E2 regulates NF-κB DNA binding activity in vivo and in vitro. Hormone-dependent breast tumors undergo regression

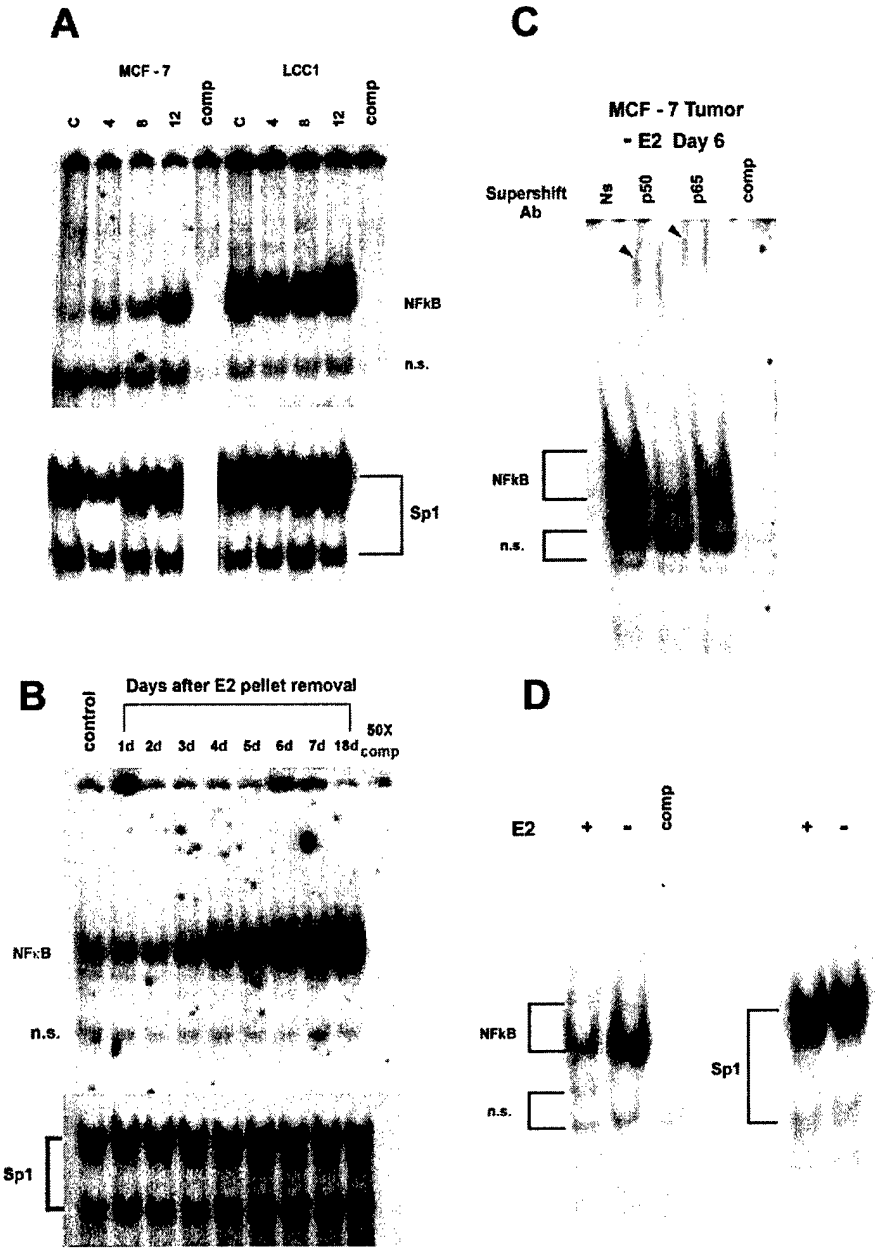

FIG. 2. E2 regulates NF-κB DNA binding in vitro and in vivo. (A) Nuclear extracts (5 μg) from MCF-7 and MCF-7/LCC1 cells cultured in DMEM (lanes C) or in E2-free medium for the indicated number of days were subjected to EMSA with the NF-κB oligonucleotide. A 10-fold excess of cold competing oligonucleotide (comp) preferentially competes the specific NF-κB complexes. n.s., nonspecific band. The lower panel shows a gel shift of the Sp1 consensus oligonucleotide with the same extracts to control for extract loading. (B) MCF-7 tumors were grown in ovariectomized nude mice implanted with an E2 release pellet. Tumors were obtained prior to E2 pellet removal (control) and at the indicated times following pellet removal. Nuclear extracts were incubated with a labeled NF-κB oligonucleotide and subjected to EMSA as described in Materials and Methods. A 50-times concentration of cold oligonucleotide (comp) was used to compete specific binding. In the lower panel the same extracts were assayed for binding to the consensus Sp1 element. (C) Tumor extracts from day 6 after the E2 pellet removal described above were used for supershift analysis with antibodies (Ab) against p50 and p65. Arrowheads indicate supershifted complexes. Ns, normal serum. (D) MCF-7 cells cultured in stripped medium without E2 for 10 days were treated with E2 or vehicle (−) for 3 days. Nuclear extracts were isolated, and 5 μg was subjected to EMSA with the NF-κB site. Protein binding to the Sp1 element from the same extracts is shown in the panel on the right.

after E2 removal by ovariectomy or following antiestrogen therapy as the result of programmed cell death of a large majority of the cells (29, 44). A subset of these cells will escape apoptosis as a result of acquisition of hormone independence and/or antiestrogen resistance. Since steroid hormones are known to modulate the levels of NF-κB activity, we tested the effects of growth in E2-free medium on the NF-κB DNA binding activity of MCF-7 and LCC1 cells. Figure 2A shows that

NF-κB activity began to increase in MCF-7 cells within 4 days after the medium was replaced with E2-free medium and continued to rise over the 12-day period studied. Thus, NF-κB activity in ER^+ MCF-7 breast cancer cells is highly responsive to removal of E2 in vitro. As expected, LCC1 cells have a high constitutive level of NF-κB DNA binding activity, which underwent a slight increase within 12 days following culture in E2-free medium. In order to determine whether this increased level of NF-κB activity also occurs in MCF-7 tumors in vivo, we grew MCF-7 tumors in nude mice implanted with E2 release pellets and isolated nuclear extracts from a solid tumor every day for 7 days and again at 18 days after pellet removal. The EMSA in Fig. 2B shows that, compared with an that of an actively growing tumor in an animal implanted with an E2 release pellet, NF-κB activity began to increase within 3 days following E2 pellet removal and rose continuously to a maximum within the 7-day period. As the tumors regressed, MCF-7 NF-κB DNA binding activity remained elevated in nuclear extracts for 18 days after E2 pellet removal. To profile NF-κB complexes in vivo from E2-depleted MCF-7 xenografts, we similarly assayed DNA binding in nuclear extracts from regressing MCF-7 cell tumors. Supershift analysis of nuclear extracts from the MCF-7 tumor excised at day 6 following E2 release pellet removal (Fig. 2C) shows again that although the NF-κB activity was primarily p50/p50 (or possibly p50/p52), an anti-p65 reactive complex was also present. Thus, both NF-κB complexes are also detectable in regressing tumors and could potentially participate in the evolution of the E2-independent phenotype. Taken together, these results show that E2 removal both in vivo and in vitro is a potent stimulus in breast cancer cells of NF-κB activity, which might then contribute to E2 independence. One interpretation of this result is that E2 removal results in survival of cells with high endogenous levels of NF-κB rather than an induction of NF-κB activity. In order to address this issue, we tested the reversibility of the effect of E2 withdrawal on NF-κB DNA binding. MCF-7 cells were grown in E2-free medium for 10 days and then treated with vehicle or E2 for 72 h. The results in Fig. 2D demonstrate that, as expected, NF-κB activity was high in MCF-7 cells cultured without E2. In contrast, levels were strongly reduced in cells which had be reexposed to E2, thus indicating regulation of NF-κB binding rather than an altered composition of the cell population.

Expression of ER proteins in MCF-7 sublines. Previous work by Nakshatri et al. (36) showed that loss of ER expression correlated with the acquisition of constitutive NF-κB activity. As LCC1 cells are E2 independent, it was possible that these cells had in fact begun to downregulate ER expression, thus resulting in higher levels of NF-κB activity. We (this work) and others (11, 17) have reported that E2 can modulate NF-κB activity; thus, alterations of ER levels in the presence or absence of E2 might indirectly affect NF-κB activity. In order to determine the relative expression levels of both of the ERs and to see whether E2 alters these levels, we performed immunoblot analysis of LCC1 cells, MIII cells, MCF-7(early) cells, and MCF-7 cells of undetermined passage treated with E2 or vehicle for 72 h. Figure 3 shows that, as expected, all cell lines express the 68-kDa ERα. Interestingly, LCC1 cells express a slightly higher level of ERα than the other cells. Similarly, immunoblotting for ERβ indicated that all of the cell lines

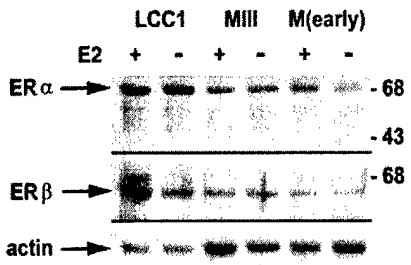

FIG. 3. Immunoblot analysis of ER proteins. Twenty micrograms of whole-cell protein extract from MCF-7 and derived sublines treated with E2 or vehicle for 72 h was separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies against ERα and ERβ. The expression of actin was used as an internal loading control. Numbers on the right indicated molecular masses in kilodaltons.

expressed a 66-kDa protein corresponding to ERβ. Again, levels appeared elevated in LCC1 cells. There was no indication that either of the ERs was subject to E2 regulation in MIII or MCF-7 cells; however, E2 reproducibly increased both ERα and ERβ of MCF-7(early) cells and the level of ERβ in LCC1 cells above levels in vehicle-treated cultures. Thus, despite this marked increase in constitutive NF-κB activity in LCC1 cells, they retain high-level expression of ERα and ERβ.

Estrogen regulates Bcl-3 protein expression. The supershift experiments described above indicated that the NF-κB complex in LCC1 cells is composed primarily of p50, which in itself is transcriptionally inactive; however, activation of the transcriptional function of this complex has been shown to occur through complex formation with Bcl-3 (31). If p50 complexes are to be active in tumors following E2 removal, the accessory factor Bcl-3 might contribute to this activity. To investigate this, we subjected protein extracts from MCF-7 tumors to immunoblot analysis at intervals after removal of the E2 release pellet. The results in Fig. 4A show that control tumors contained low levels of Bcl-3, while the levels rose significantly within 1 day after E2 removal and remained elevated for the balance of the study. To assess whether Bcl-3 expression induced by E2 removal was principally due to the absence of E2 or was secondary to other effects on the tumor milieu, we analyzed extracts from LCC1 cells cultured in E2-free or E2-supplemented medium over several days. The results in Fig. 4B show that culture of LCC1cells in the absence of E2 increased the expression of Bcl-3 within 1 day compared with culture in E2-supplemented medium. The differential expression was already maximal by 2 days and remained so throughout the 4-day study period. Since NF-κB DNA binding is constitutively higher in E2-independent LCC1 cells and MDA-MB-231 cells than in MCF-7 cells and MIII cells in the early stage of E2 independence, we wished to determine whether basal levels of Bcl-3 might follow the same pattern. Figure 4C shows a Western blot analysis of Bcl-3 levels in extracts from early- and late-passage MCF-7 cells, MIII cells, LCC1 cells, and several ER^- cells. The results indicate that levels of Bcl-3 are highest in the E2-independent lines, including LCC1 cells. A second comparison of Bcl-3 levels in MCF-7(late), MCF-7(40F) and MDA-MB-468 cells (Fig. 4D) shows that, unexpectedly, MCF-7(40F) cells express Bcl-3 at approximately the same level as

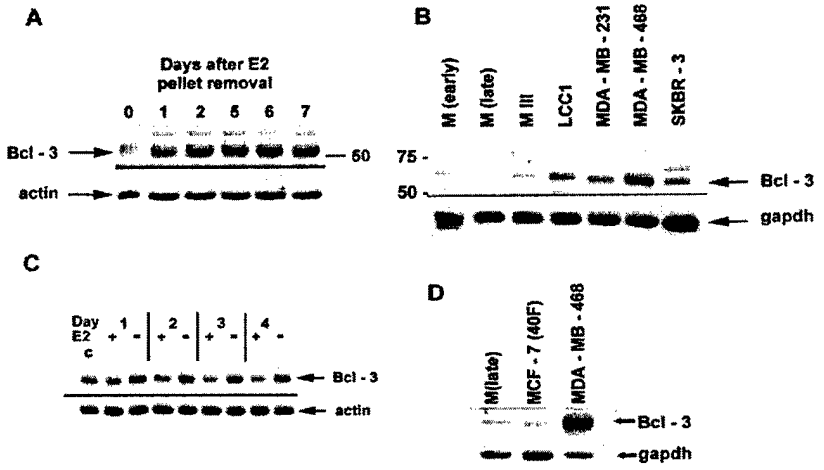

FIG. 4. Bcl-3 is regulated by E2 in breast cancer cells in vivo and in vitro. (A) Bcl-3 expression in MCF-7 breast cancer tumors was determined by immunoblotting of 15 μg of tumor protein extracts on the indicated days after E2 release pellet removal. The molecular mass in kilodaltons is shown on the right. (B) Basal expression of Bcl-3 in E2-dependent and -independent breast cancer cells. Whole-cell protein extracts were isolated from the indicated breast cancer cell lines grown in appropriate phenol red-containing medium. In all experiments,15 μg of each extract was subjected to immunoblot analysis with the Bcl-3 antibody and either actin or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a protein loading control. (C) Immunoblot to detect Bcl-3 expression in vitro in the presence and absence of E2. LCC1 cells were cultured in DMEM or E2-free medium supplemented with E2 or vehicle as described in Materials and Methods for the indicated times. Lane c, control consisting of extracts from cells cultured in unstripped medium containing phenol red. (D) Comparison of Bcl-3 expression in the indicated cell lines as described for panel C.

MCF-7 cells. However, it is important to note that MCF-7(40F) cells were selected not for E2 independence but rather for adriamycin resistance and therefore do not necessarily represent a natural stage in hormone progression. Moreover, we cannot rule out that regulation of Bcl-3 expression in other ER^- cell lines is accomplished by the same mechanism as in ER^+ cell lines.

Bcl-3 augments MCF-7 tumor growth but not E2 independence. Bcl-3 has recently been shown to stimulate growth as well as provide a survival function in some cells (35, 47, 55). The results described above clearly show that p50/50 (and possibly p50/p52) complexes predominate in LCC1 cells, and the increased expression of Bcl-3 suggests that this protein could play a role in E2 independence. To assess the sufficiency of Bcl-3 expression in conferring E2 independence, we stably transfected MCF-7(early) cells with a FLAG-tagged Bcl-3 expression construct. Figure 5A shows an immunoblot of lysates from three pooled MCF-7(FLAG-Bcl-3) clones and three pooled MCF-7(FLAG) control clones reacted with anti-FLAG to detect transfected protein (upper panel) and anti-Bcl-3 (second panel) in order to compare the relative levels of Bcl-3 in these clones. Bcl-3 expression has been associated with induction of the cyclin D1 gene, and immunoblot analysis of the same nuclear extracts with an anti-cyclin D1 antibody showed that the constitutive level of cyclin D1 expression was increased in the transfected cells (third panel). Nuclear extracts from these pooled clones were also used for an EMSA to detect NF-κB DNA binding activity. Figure 5B shows that, consistent with its ability to interact with p50, the p50 complex was augmented in MCF-7(FLAG-Bcl-3) clones compared with controls when equal amounts of nuclear extract were assayed, suggesting a possible stabilization of the p50 DNA binding complex. We then used both MCF-7(FLAG-Bcl-3) and MCF-7(FLAG) clones to generate tumors in ovariectomized nude mice implanted with an E2 release pellet as described in Materials and Methods. The growth of control tumors [MCF-7(FLAG)]and MCF-7(FLAG-Bcl-3) tumors was monitored over 36 days, and the results are shown in Fig. 5C. Eleven of the 15 sites injected with MCF-7(FLAG-Bcl-3) cells produced tumors. On the other hand, only 4 of 12 control sites formed tumors over the same time period. Of the tumors that formed, the mean volumes (± SE) were 258 ± 45 mm^3 for MCF-7(FLAG-Bcl-3) tumors and 67 ± 24 mm^3 for MCF-7(FLAG) tumors. Tumor regression was monitored after E2 release pellet removal until the tumor was 50% of its original size at pellet removal. If Bcl-3 conferred E2 independence, we expected that MCF-7(Bcl-3) tumors would either remain stable or continue to grow. However, comparison with control MCF-7(FLAG) tumors showed virtually identical regression rates, requiring 14 days for the latter tumors and 15.5 days for MCF-7(FLAG-Bcl-3) tumors to regress to 50% of the original tumor volume. Thus, Bcl-3 alone does not render MCF-7 tumors stable after E2 withdrawal, suggesting that Bcl-3-mediated increases in p50 and/or p52 activity are either of insufficient magnitude or cannot confer an E2-independent phenotype. In contrast, the higher rate of tumor establishment and rapid tumor growth of MCF-7(FLAG-Bcl-3) cells compared with control cells shows that Bcl-3 can augment the growth and tumorigenicity of breast cancer cells.

Induction of both p65- and p50-associated activity after E2 withdrawal in vitro and in vivo. The relative levels of p65 and p50 in nuclei from MCF-7(early) and LCC1 cells and the results in Fig. 1B suggested that there were both qualitative and quantitative differences between NF-κB complexes in the

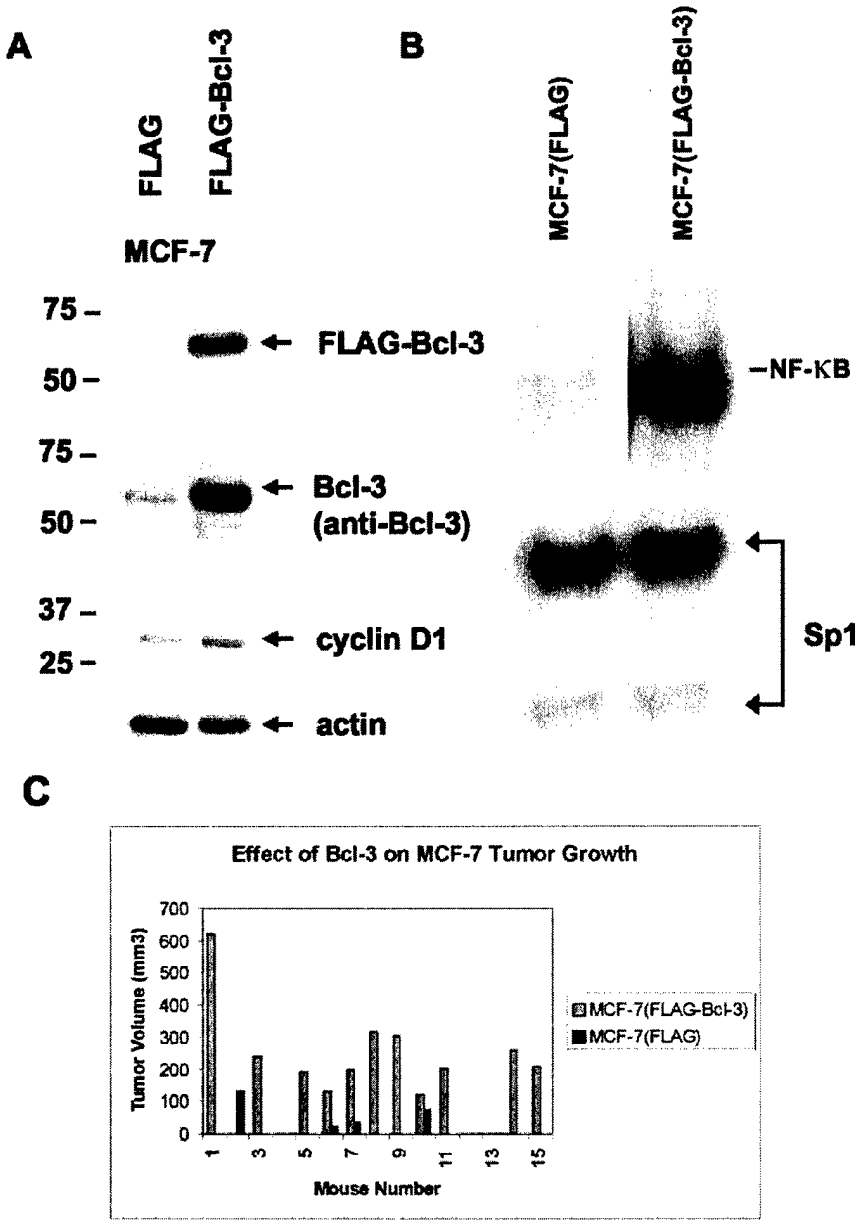

FIG. 5. Bcl-3 promotes E2-dependent MCF-7 tumor growth. (A) MCF-7 cells were transfected with either an empty FLAG vector or FLAG–Bcl-3. Shown is immunoblot analysis of cell extracts from three pooled clones of each transfectant with an anti-FLAG antibody. The lower panel shows a duplicate blot following incubation with anti-Bcl-3 to detect both endogenous and transfected protein. Sizes of molecular mass markers are shown on the left in kilodaltons. (B) EMSA to assess the effects of Bcl-3 overexpression on NF-κB DNA binding. Five micrograms of nuclear extract from pooled MCF-7(FLAG) and MCF-7(FLAG-Bcl-3) clones was used for gel shift analysis with either the NF-κB or Sp1 sequence as a probe. Samples were assessed simultaneously on the same gel. (C) Tumor volumes derived from MCF-7(FLAG) and MCF-7(FLAG-Bcl-3) cells 36 days after cell inoculation. Mice 1 to 12 were injected subcutaneously with pooled clones of control or MCF-7(FLAG-Bcl-3) on either flank. Mice 13 to 15 received only an MCF-7(FLAG-Bcl-3) inoculation.

hormone-dependent and hormone-independent cells. To characterize the NF-κB complexes in these two isolates, it was necessary to use MCF-7(early) cells which were cultured in E2-free medium, since without E2 withdrawal there was insufficient NF-κB DNA binding activity to evaluate. We performed an EMSA and supershift analysis of nuclear extracts from MCF-7(early) and LCC1 cells which, for consistency, were both derived from culture in E2-depleted medium for 12 days (Fig. 6). Supershift analysis with the p50 antibody again revealed the presence of a major complex containing p50, while the p52 antibody was unable to supershift any complex. Importantly, the p65 antibody identified a strong p65-associated complex in the LCC1 cells. By contrast, the level of p65 DNA binding activity was significantly lower in the MCF-7(early)

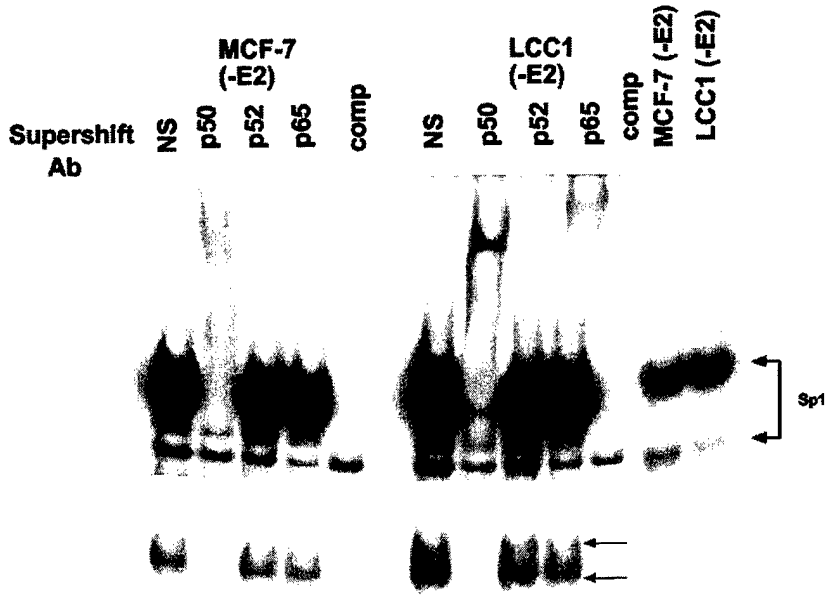

FIG. 6. Induction of both p65- and p50-associated NF-κB activity following E2 withdrawal. Nuclear extracts were collected from MCF-7(early) and LCC1 cells cultured for 12 days in phenol red-free medium with charcoal-stripped serum to induce NF-κB activity. Five micrograms of each extract was used for EMSA with the NF-κB probe and antibody supershift analysis with the indicated antibodies (Ab). The same quantity of extract was also subjected to EMSA with the Sp1 probe as a control for extract loading.

nuclear extracts. Taken together with the nuclear expression of p65 and p50 in MCF-7 and LCC1 cells, these results show that after E2 withdrawal, LCC1 cells contain significantly more p65-associated activity than do their E2-dependent counterparts.

LCC1 cells constitutively expressing IκBα revert to an estrogen-dependent tumor phenotype. In order to test the hypothesis that NF-κB activity contributes significantly to the E2-independent phenotype, we stably transfected LCC1 cells with a FLAG-tagged degradation-resistant form of the NF-κB inhibitor IκBα called $I\kappa B\alpha^{SR}$ (27). IκBα most effectively inhibits p65-containing complexes (26), although some minimal inhibition of p50/p50 activity has also be documented (30). Figure 7A shows immunoblot analysis with the anti-FLAG monoclonal antibody of four positive clones and control extracts from pcDNA3-transfected LCC1 cells. The effects of $I\kappa B\alpha^{SR}$ expression on relative levels of nuclear NF-κB complexes were tested by supershift analysis. Figure 7B shows that $I\kappa B\alpha^{SR}$ expression had little effect on the abundant, fast-migrating p50-containing complex. In contrast, the p65 complex was virtually absent from the LCC1($I\kappa B\alpha^{SR}$) cells. To confirm that any reversion of LCC1($I\kappa B\alpha^{SR}$) tumors to E2 dependence was associated with decreased nuclear levels of p65, we performed immunoblot analysis of nuclear extracts from LCC1(pcDNA3) and LCC1($I\kappa B\alpha^{SR}$) tumors. Figure 7C shows that p65 levels are markedly reduced in LCC1($I\kappa B\alpha^{SR}$) tumors compared with controls. On the other hand, both the p50 and p52 nuclear contents remained unaltered in these tumor types. The p50 antibody, which simultaneously detects p52 in extracts from cultured cells, did not detect p52 in the tumor extracts. To assess p52, we utilized a separate anti-p52 antibody, which indicated that the levels of nuclear p52 were unchanged in $I\kappa B\alpha^{SR}$-expressing tumors. Evaluation of the effects of $I\kappa B\alpha^{SR}$ on NF-κB transcriptional activity following transient transfection of the three highest-expressing clones with the 3X-NF-κB reporter gene or the same reporter gene containing a mutant NF-κB response element showed that transcriptional activity in the three LCC1($I\kappa B\alpha^{SR}$) clones ranged from 20- to 100-fold less than that in the control LCC1(pcDNA3) clones (Fig. 7D). We then inoculated three pooled LCC1(pcDNA3) clones and pooled LCC1($I\kappa B\alpha^{SR}$) clones 1, 3, and 4 into the contralateral sides of ovariectomized nude mice implanted with an E2 release pellet. Tumors from both groups grew at various rates under these conditions of hormone replacement, and there was no significant difference in the final tumor volumes [mean ± standard deviation, 183 ± 88 mm^3 for LCC1(pcDNA3) tumors and 160 ± 88 mm^3 for LCC1($I\kappa B\alpha^{SR}$) tumors]. Tumor volumes were measured 2 weeks after E2 pellet removal, and the percent regression was calculated. Figure 7E is a histogram showing the results of this analysis. While LCC1(pcDNA3) tumor volumes either remained stable or continued to grow after pellet removal, LCC1 ($I\kappa B\alpha^{SR}$) tumors underwent significant regression. In cells engaged in apoptosis through the mitochondrial death pathway, the Bax protein undergoes a conformational change exposing its otherwise buried N terminus associated with mitochondrial translocation (37). Figure 7F depicts sections from LCC1(pcDNA3) and LCC1($I\kappa B\alpha^{SR}$) tumors immunostained with an antibody against the N terminus of Bax, showing high levels of translocated Bax in the LCC1($I\kappa B\alpha^{SR}$) tumors but not in control tumors. Moreover, detection of DNA fragmentation in apoptotic cells by using ISEL showed that LCC1($I\kappa B\alpha^{SR}$) tumors contained large numbers of apoptotic nuclei, while LCC1(pcDNA3) tumors had almost none. Thus, NF-κB inhibition is sufficient to restore

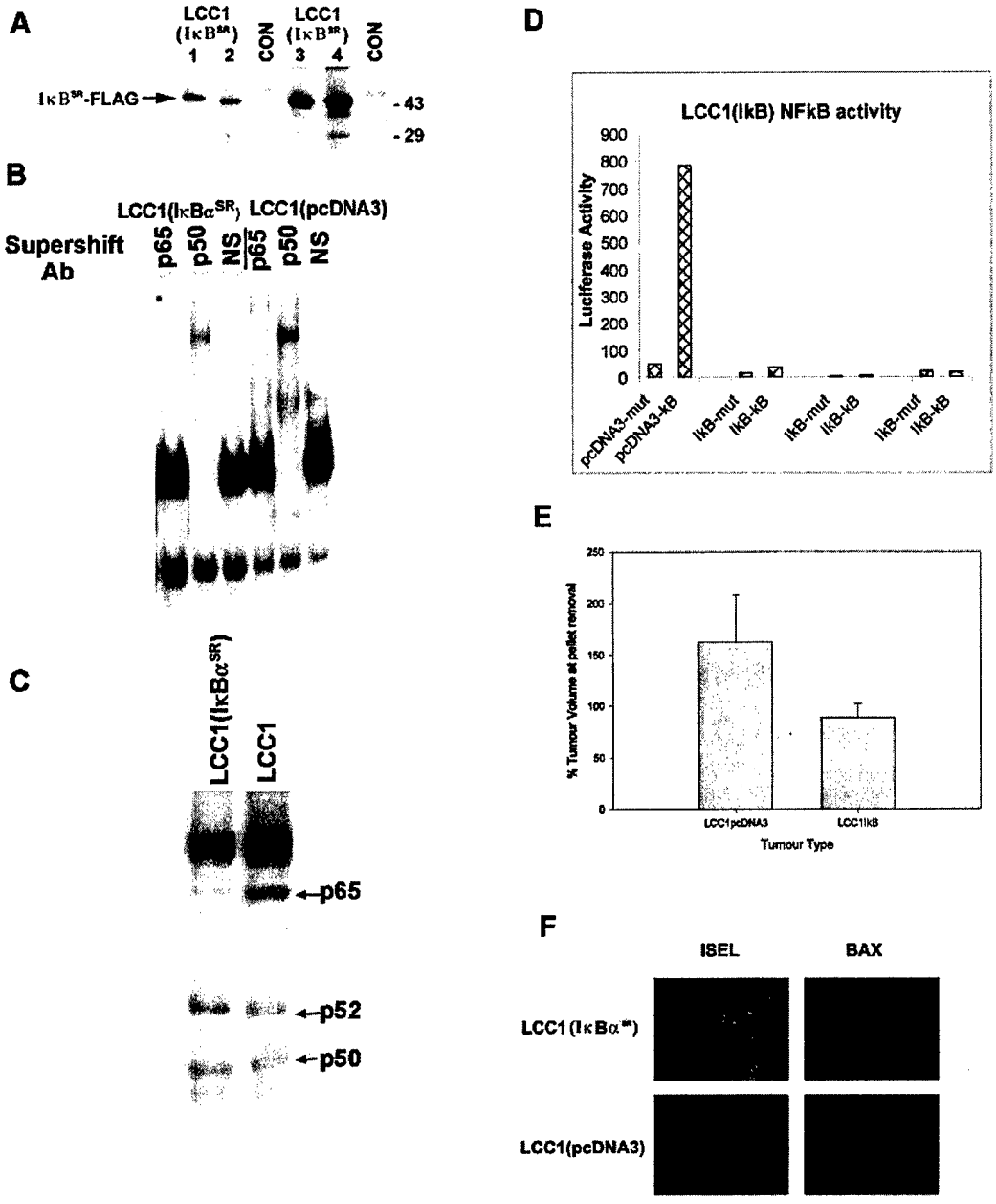

FIG. 7. MCF-7/LCC1 cells constitutively expressing IκBα^{SR} lose p65/p50 activity and revert to an E2-dependent tumor phenotype. (A) Immunoblot analysis to detect FLAG-tagged IκBα^{SR} expression in stable LCC1 clones with an anti-FLAG monoclonal antibody. Clones are designated 1 to 4. CON, control. (B) Five micrograms of nuclear extract from LCC1(pcDNA3) and LCC1(IκBα^{SR}) cell cultures was subjected to EMSA and antibody (Ab) supershift analysis as described in Materials and Methods. NS, normal serum. (C) Nuclear extracts (10 μg) from LCC1(IκBα^{SR}) and LCC1(pcDNA3) tumors were analyzed by immunoblotting for expression of p65, p50, and p52. The p50 antibody did not identify a p52 band from these extracts, and therefore a p52-specific antibody was used to assess changes in expression between clones. (D) NF-κB activity was evaluated in LCC1(IκBα^{SR}) clones 1, 3, and 4 (from left to right) following transient cotransfection of the NF-κB-luciferase and LacZ reporter genes. The results presented are the averages from two experiments, expressed in arbitrary units, and are normalized to β-galactosidase activity. (E) Three pooled clones each of LCC1(pcDNA3) and LCC1(IκBα^{SR}) cells were inoculated into ovariectomized nude mice implanted with an E2 release pellet. The histogram shows the percent regression of LCC1 (n = 11) and LCC1(IκBα^{SR}) (n = 9) tumors following removal of the release pellet. Bars represent standard errors. (F) Sections of LCC1 and LCC1(IκBα^{SR}) tumors were subjected to ISEL to detect free 3′-OH DNA ends in apoptotic nuclei or immunostained with an antibody against the NH_2 terminus of Bax to detect activated mitochondrial Bax.

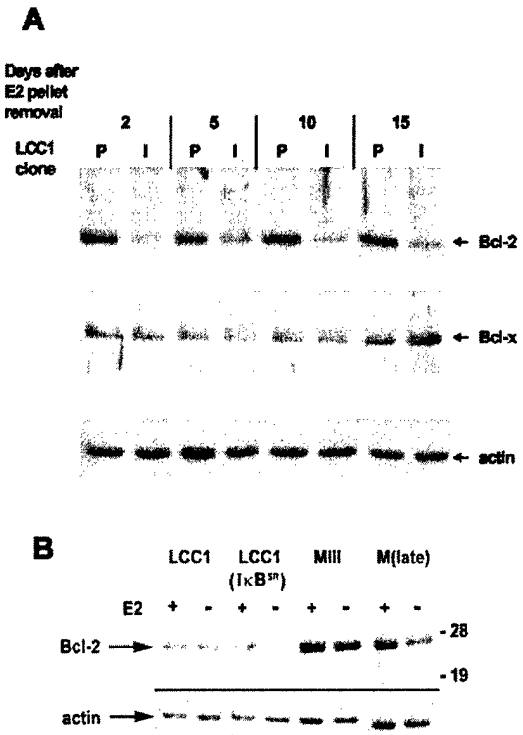

FIG. 8. Expression of IκBα^{SR} downregulates Bcl-2 expression. (A) Whole-cell extracts from LCC1(pcDNA3) (lanes P) and LCC1(IκBα^{SR}) (lanes I) cell tumors at different days following E2 pellet removal were analyzed by immunoblotting for expression of Bcl-2. Blots were stripped and reacted with antibody against Bcl-x. Loading of the gel was controlled for by reactivity with an actin antibody. (B) LCC1 cells stably transfected with pcDNA3 or IκBα^{SR} together with MIII and M(late) cells were cultured in E2-free medium for 10 days and then treated with E2 or vehicle for 48 h. E2 regulation of Bcl-2 expression in vitro in the MCF-7-derived cells and clones was assessed by immunoblotting of 15 μg of cell extract. Actin was used as an internal loading control for all blots. Molecular masses in kilodaltons are shown on the right.

an E2-dependent phenotype to LCC1 tumors, rendering them apoptotic after E2 removal through a mitochondrial pathway of cell death involving Bax translocation.

Effect of NF-κB inhibition on E2-regulated genes. Recent work has shown that expression of both of the antiapoptotic proteins Bcl-2 and Bcl-x is induced by NF-κB in neurons (10, 51). Since LCC1(I-κBα^{SR}) tumors undergo apoptotic regression following E2 withdrawal, we tested whether the reduced NF-κB in these cells was associated with a reduction in Bcl-2 and/or Bcl-x. We first considered the expression of these proteins in the respective tumors on various days following E2 release pellet removal. The results in Fig. 8A show that Bcl-2 protein levels were strongly reduced in LCC1(IκBα^{SR}) cells compared with LCC1(pcDNA3) cells for the entire period after withdrawal of E2. In contrast, Bcl-x expression in the two tumor types was indistinguishable. During the initial characterization of MIII and LCC1 cells, it was found that the expression of a number of normally E2-responsive genes was constitutively upregulated and no longer responsive to E2 (9). This was also true of another independent MCF-7 subline selected for E2-independent growth (13). E2 can also regulate expression of Bcl-2 (43, 52), although the promoter for this gene is complex and may also be regulated by mutant p53. A comparison of the expression of Bcl-2 and its regulation by E2 in vitro is shown in Fig. 8B. Unlike for several other E2-regulated gene products, LCC1 cells contain lower levels of Bcl-2 than do parental MCF-7 cells. This expression is refractory to E2 regulation. As observed in the tumors, stable expression of IκBα^{SR} further reduced Bcl-2 expression in vitro, and this was accompanied by a weak reinstatement of E2 regulation. Importantly, we have previously shown that constitutive Bcl-2 expression is sufficient to prevent regression of MCF-7 tumors in nude mouse xenografts (44). Taken together, these results suggest that the reduction in Bcl-2 expression in LCC1(IκBα^{SR}) tumors could contribute to the E2 withdrawal-induced apoptosis and subsequent regression.

DISCUSSION

The events which precede the loss of E2 dependence and a more tumorigenic phenotype in breast cancer cells are not understood. The LCC1 subline of the E2-dependent human breast cancer cell line MCF-7 was selected under conditions of E2 depletion, resulting in an experimental model of progression in that these cells grow in a hormone-independent manner but retain wild-type levels of ER expression. Apart from the observation that some E2-regulated genes are constitutively expressed in these cells, little is known about the cellular events that contribute to the loss of E2 dependence. While it is clear that NF-κB activity rises in the context of carcinogen-induced mammary epithelial cell transformation (28), in general there is also a marked increase in constitutive NF-κB activity in ER$^-$ cells compared with ER$^+$ cells (36). Using E2-independent LCC1 cells, the work described here shows that an increase in constitutive NF-κB activity occurs in cells selected for E2 independence prior to loss of ER expression. Moreover, inhibition of this NF-κB activity is sufficient to confer sensitivity to estrogen removal on LCC1 tumors through a mitochondrial death pathway. This observation also clearly indicates that LCC1 cells have not sustained damage to their apoptotic machinery during progression toward E2 independence. In contrast to K-BALB murine fibrosarcoma cells and mouse mammary tumor cells, in which tumorigenicity was completely blocked by inhibition of NF-κB activity (7, 22), at least in animals supplemented with E2, LCC1(IκBα^{SR}) cells were able to form tumors as well as control cells. Thus, inhibition of NF-κB activity through IκBα does not interfere with E2-driven tumorigenicity.

The process of selection requires epigenetic changes which render some cells resistant to the selective pressure. Therefore, to investigate the mechanism by which this occurs, we have analyzed the consequence of E2 removal on NF-κB activity both in vitro and in tumors. The reduction in E2 levels following removal of the E2 pellet in ovariectomized athymic mice and culture in E2-free medium results in a rapid increase in NF-κB levels in MCF-7 cells. The kinetics were fastest in vivo, which may reflect E2 clearance through metabolism. Additionally, cells in culture were exposed to estrogenic phenol red prior to removal to phenol red-free stripped medium, and the clearance rate of phenol red in these cells. Several reports have

previously shown that the ligand-bound ER is involved in reciprocal regulation with NF-κB (reference 17 and references therein). Both ERα and the ERβ can inhibit NF-κB-dependent transcription in a manner involving the ligand binding domain of the receptor but not the squelching of coactivator proteins (11). Discreet parts of the ER ligand binding domain surface are required for this transrepression of NF-κB activity, which are separate from those involved in transactivation (53). Estrogen has been clearly demonstrated to repress NF-κB activity, and correspondingly, NF-κB induction follows E2 withdrawal. Estrogen removal over the time course of our experiments in vitro is only cytostatic for MCF-7 cells. Given this and the fact that NF-κB activation is reversed by reintroduction of E2 in vitro, it is unlikely that the induction of NF-κB DNA binding seen after removal of E2 in vivo is the result of selection for cells with higher basal levels of NF-κB. Moreover, NF-κB is a positive regulator of cell growth (12), thus arguing against subpopulations of MCF-7 cells containing constitutively elevated NF-κB activity.

Since the predominant NF-κB complex in MCF-7 and LCC1 cells is either a homodimeric complex of p50 or a heteromeric complex of p50 and p52, activation after hormone removal would not be expected to yield a strong transcriptional response. Our finding that the level of the p50-coactivating protein Bcl-3 is increased after E2 removal provides a mechanism by which E2 withdrawal induces p50-associated NF-κB activity in breast cancer cells that do not contain high levels of the alternate transcriptionally active partner, p65. The observation that Bcl-3 increases the E2-dependent growth of MCF-7 cells may be due in part to the reported ability of Bcl-3/p50/p52 complexes to induce cyclin D1 (23, 55) as well as stimulate AP-1-mediated transactivation and cellular proliferation (35). Although the Bcl-3 regulatory regions contain NF-κB enhancer elements (40) that are responsive to NF-κB (8, 10), this mechanism cannot account for the rapid increase in Bcl-3 protein expression observed both in vitro and in vivo after E2 withdrawal, suggesting that Bcl-3 expression may be more directly under control of the ER.

The present data indicate that p50 and p65 are differentially expressed during progression and that early in this process NF-κB activity increases and is composed primarily of p50/p50 (and possibly p50/p52), along with a smaller increase in p50/p65. Highly malignant ER^- cells have both p65/p50 and p50 (p50/p52)-associated NF-κB DNA binding activity. Evidence suggests that the p65 protein plays a critical role in tumorigenesis (22). Nuclear extracts from ER^+ MCF-7 cells of limited passage express only trace levels of p65 and comparatively low levels of p50. LCC1 nuclei contain slightly elevated p65, although the level remains significantly lower than that in the ER^- cell lines. However, LCC1 p50 nuclear expression is essentially similar to that in the E2-independent breast cancer cell lines. Together with the finding that basal Bcl-3 expression in LCC1 cells is also higher than that in the parental MCF-7 isolate, these results can account for the higher constitutive level of both NF-κB complexes in LCC1 cells. The expression pattern of p65 and p50/p52 in breast tumors is unclear. It has been suggested that breast cancer cell lines but not tumors express p65. While Cogswell et al. (16) detected only low levels of p65 protein in a panel of four breast tumor samples, only one of these was ER^-. Supershift analysis was done on an ER^+ tumor which, in agreement with our results, showed primarily p50-associated NF-κB DNA binding activity. Sovak et al. (50) detected nuclear p65 in 15 of 23 tumor samples, although that study did not differentiate on the basis of ER status. Thus, it appears that NF-κB proteins and complexes are differentially present in breast cancer cells, which, at least in cell lines, correlates with both ER expression and the stage of progression. One of the most important outcomes of this study is that it describes an underlying reason for why primary breast tumors with functional ERs may be marginally or not at all E2 dependent. With respect to both Bcl-3 expression and NF-κB activity, like LCC1 cells and MDA-MB-231 cells, tumors that display elevated Bcl-3 could be either ER^+ or ER^-. Another variable that could affect these parameters in tumors that are either truly E2 dependent or just E2 responsive is the level of circulating E2 in a patient at the time of biopsy. Based on our results, Bcl-3 and NF-κB activity would not be predicted to correlate simply with ER status in the absence of any other information about the tumor or patient. Instead, ER expression combined with Bcl-3 and differential NF-κB activity might predict the actual hormone-dependent status of the tumor and prove to be a useful marker for progression.

The targets of NF-κB are numerous and varied, ranging from cytokines in the immune system to cell adhesion molecules, transcription factors, a variety of enzymes, and certain survival proteins (42). The last group includes the antiapoptotic protein Bcl-xL as well as Bcl-2, whose expression has been shown to be activated by NF-κB in neuronal cells (10, 51). We have previously demonstrated that Bcl-2 is positively regulated by E2 in MCF-7 cells (52), although this regulation appears to be absent in LCC1 cells and basal levels of Bcl-2 are lower than in related MCF-7 cells. We have also shown that Bcl-2 expression is sufficient to prevent E2 withdrawal regression of MCF-7 tumors in nude mice (44). Thus, a further decrease in expression of Bcl-2 in LCC1($I\kappa B\alpha^{SR}$) cells would likely contribute to the acquired E2 sensitivity of LCC1 ($I\kappa B\alpha^{SR}$) tumors by facilitating the apoptotic response following the death signal constituted by E2 release pellet removal. The fact that Bcl-2 but not Bcl-x was reduced by $I\kappa B\alpha^{SR}$ expression suggests that p65/p50 complexes may positively regulate Bcl-2 expression while Bcl-x is regulated either by different NF-κB complexes or by other means in these cells. The IAP protein hIAP1 (18) has been shown to be induced by NF-κB (reference 42 and references therein), and while our preliminary findings indicate higher levels of both hIAP1 and hIAP2 in those breast cancer cells containing elevated constitutive NF-κB activity (data not shown), the role of these proteins in hormone independence requires further investigation.

The work presented here demonstrates that NF-κB complexes play distinct roles in E2-dependent and -independent growth and survival of breast cancer cells. Complexes of p50 (and possibly p52) and Bcl-3 in LCC1 cells promote the growth of LCC1 cells in an E2-dependent manner and, although this was not directly tested here, likely contribute to growth promotion in the presence of an appropriate survival signal in E2-independent cells as well. The majority of NF-κB DNA binding activity is composed of p50 dimers; however, expression of Bcl-3, which is required for the transcriptional activity of these complexes, is insufficient to confer E2 independence to MCF-7 cells. Thus, p65-containing complexes in LCC1 cells,

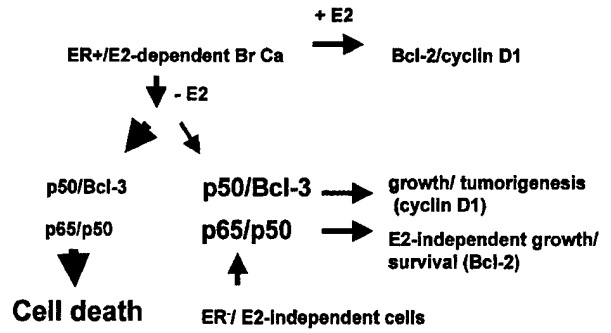

FIG. 9. Proposed model of induction of NF-κB-mediated estrogen-independent growth and survival following estrogen withdrawal from ER^+ breast cancer cells. E2 provides growth and survival signals to ER^+ breast cancer cells. E2 withdrawal induces NF-κB activity which in most cells is not sufficient to support E2-independent growth, and the tumor regresses. However, some cells could persist as E2-independent variants as a result of adequate expression of differential NF-κB activity. ER^- breast cancer cells have constitutive p65 and p50 activity. See text for details.

either alone or in concert with p50 dimer/Bcl-3 complexes, clearly have a critical role in conferring the E2-independent phenotype. Supporting this hypothesis is the observation that expression of $I\kappa B\alpha^{SR}$ protein clearly reduced nuclear p65 levels in LCC1 tumors but had little effect on p50 or p52. Moreover p65 complexes were virtually undetectable by supershift analysis in LCC1($I\kappa B\alpha^{SR}$) cells. The crystal structure of the IκBα:NF-κB complex has been resolved, revealing a stable and extensive interaction between IκBα and p65. The outcome of this interaction is a critical change in the conformation of p65, resulting in allosteric inhibition of DNA binding as well as masking of the nuclear localization signal in p65 (26). Although IκBα can bind p50 homodimers, it does so with a 60-fold-lower affinity than in its interaction with p65/p50 (30). Thus, the data presented here, as well as the fact that all ER^- or E2-independent cell lines have high levels of p65/p50 NF-κB activity, support the hypothesis that breast cancer cell progression requires increased p65/p50 activity.

Our results together with those in the literature suggest the model in Fig. 9, which indicates pathways of growth and survival in ER^+ breast cancer cells in both the presence and absence of E2. E2 provides dual growth and survival signals at least in part by increasing cyclin D1 and Bcl-2 protein levels. We surmise that the increase in Bcl-3 after E2 withdrawal could also contribute to increased proliferative rates, based on the increased rate of tumor growth generated by Bcl-3-overexpressing cells. This increased proliferation is insufficient, however, to prevent the overall regression of the tumor over time. Thus, our model suggests that while most E2-dependent cells will undergo cell death after E2 withdrawal in vivo, a subset with adequate levels of p50/p52/Bcl-3 and p50/p65 will both grow and survive, thus establishing the E2-independent phenotype. It is possible that these two events may not occur simultaneously in tumor cells and that the immediate induction of Bcl-3 after E2 withdrawal may be sufficient over the short term to maintain the tumor while the proliferative rate exceeds the rate of apoptosis. This could provide adequate time for other signaling pathways, such as ErbB2, which is also induced after E2 withdrawal in MCF-7 cells (data not shown), to induce p50/p65-associated activity (6, 57). Studies to investigate the possible role of ErbB receptors in this process are under way.

The ability of IκBα to inhibit E2-independent growth in this study shows that NF-κB activation may be the most critical early event following E2 withdrawal resulting in breast cancer progression. Therefore, in addition to representing a potential therapeutic target in ER^- breast cancer as proposed by Biswas et al. (7), NF-κB inhibition may well prove to be effective in obviating progression. Ironically, a reduction in circulating E2 levels (or spontaneous loss of ER expression) in individuals with hormone-dependent breast cancer would directly precipitate the early induction of NF-κB activity, which could confer E2-independent growth and survival to these cells.

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Discriminatory Mining of Gene Expression Microarray Data

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Abstract

Recent advances in machine learning and pattern recognition methods provide new analytical tools to explore high dimensional gene expression microarray data. Our data mining software, VISual Data Analysis for cluster discovery (VISDA), reveals many distinguishing patterns among gene expression pro...les, which are responsible for the cell's phenotypes. The model-supported exploration of high-dimensional data space is achieved through two complementary schemes: dimensionality reduction by discriminatory data projection and cluster decomposition by soft data clustering. Reducing dimensionality generates the visualization of the complete data set at the top level. This data set is then partitioned into subclusters that can consequently be visualized at lower levels and if necessary partitioned again. In this paper, three di¤erent algorithms are evaluated in their abilities to reduce dimensionality and to visualize data sets: Principal Component Analysis (PCA), Discriminatory Component Analysis (DCA), and Projection Pursuit Method (PPM). The partitioning into subclusters uses the Expectation-Maximization (EM) algorithm and the hierarchical normal mixture model that is selected by the user and veri...ed "optimally" by the minimum description length criterion. These approaches produce di¤erent visualizations that are compared against known phenotypes from the microarray experiments. Overall, these algorithms and user-selected models explore the high dimensional data where standard analyses may not be su¢cient.

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Keywords

Computational bioinformatics, gene microarrays, ...nite normal mixture, cluster visualization and selection, machine learning.

I. Introduction

With gene expression microarrays, the relative expression levels in two or more mRNA populations derived from tissue samples can be assayed for thousands of known sequenced genes simultaneously [1], [2]. Thus, this assay makes them an e¢cient and cost-e¤ective tool for large scale analysis of gene expression in recent years. Their design is composed of a platform (glass slide, nylon ...lter, or chip) with bounded cDNA fragments or oligonucleotides that code for either known gene sequences or Expressed Sequence Tags (ESTs). Microarrays have been used to classify clinical samples, to investigate the mechanism of drug action, to examine the e¤ects of drugs on gene expression in yeasts, and to identify and validate novel therapeutics for cancer patients [1][3][4]. Still hidden links remain between genes and the biology of cancer. They, however, may be revealed through a large scale of gene expression analysis of normal and cancer cells. Speci...cally, an altered gene expression pattern in the malignant tissues can determine their phenotypes, e.g., drug responses, growth proliferation rate, angiogenesis, and metastases. Microarrays allow mass measurements of gene expression to occur, but the tools to analyze the data are not well developed [5]. Because the number of dimensions in a microarray data set could reach from hundreds to tens of thousands, the development of these analytical tools is crucial.

Advances in microarray technologies have enabled investigators to explore the dynamics of transcription on a molecular scale. The current challenge is to extract useful and reliable information out of these large data sets. A common and ...rst approach is cluster analysis. The primary objective of cluster analysis is to group genes that have comparable patterns of variation. This approach is valuable for reducing the complexity of large data sets and for identifying predominant patterns within

the data. However, additional methods are needed to extract information about individual genes from these large data sets.

The gene expression pro...le (mRNA), one of the molecular signatures (DNA, mRNA, and protein), is a snapshot of the malignant and proliferative mechanism behind cancer. The representation of each sample's pro...le is described as a point in a d-dimensional gene expression space in which each axis represents the expression level of one gene. The presence of well-separated sample groups implies that the representations of samples within the same group are close to each other in this gene expression space but distant from those of other samples. Thus, the representations of phenotype-related samples form clusters.

The research plan is divided into three major steps: cluster discovery, gene selection, and phenotype prediction. Cluster discovery detects previously unrecognized tumor subtypes [5]. Gene selection identi...es the most relevant gene subset involving the biological process that generates the patterns. Phenotype prediction assigns unknown tumor sample to known tumor classes [5]. The main challenge, however, is that the microarray data is high-dimensional, multi-modal, and lacking in prior knowledge.

Data clustering is a process of grouping input data points with similar features in the multidimensional space; the algorithms are being investigated for long time. The most common hierarchical clustering method often used by biologists for data clustering is dendrogram [6]. At the end of the analysis the data points are arranged into a phylogenetic tree, the level of similarity of two pairs is represented by the length of the branch. However, even though the hierarchical clustering is simple and straight forward, it is designed to re‡ect true hierarchical tree structure and that is not the way in which microarray data is generated. It is very important to include more biological information rather than rigidly clustering data points. Hierarchical clustering may fail to group data points in the right way because it is greatly in‡uenced by local condition and has no opportunity of evaluating the global structure. Support Vector Machines (SOM) attempts to search for relevant patterns by ...rst imposing structure on the data with nodes that are expected to eventually move to the center of each cluster,

and then updating the structure map in each iteration based on a data point randomly selected from the data set [7]. The result ends up gathering similar samples in the same cluster. Studies of using SOM to cluster genes have been done by Whitehead Institute/MIT [7]. Under unsupervised situation, the success of this approach partially depends on the initialization of the map structure, e.g. number of nodes and di¤erent geometries. Without data modelling, SOM lacks criteria for validation of cluster structure, e.g. whether the number of clusters is optimal.

A gene clustering method based on graph theoretic techniques is developed for the situation that the clusters are not assumed to be hierarchically structured [8]. The cluster information is mapped to an undirected graph where each clique in the graph indicates a cluster. It is assumed in the model that the input data contain underlying cluster structure contaminated by random errors. Through applying this clustering algorithm, with high probability, the cluster structure can be recovered by removing the random errors from the input data. However, the algorithm is developed for gene clustering, in which the input data have much lower dimensionality than the microarray data we have (about 20 vs. 500 » 8000), its capability of handling high dimensional data is uncertain. In addition, the microarray data have signi...cantly large overlaps among clusters resulting from the nature of biological data. The potential application of this algorithm to microarray data is not optimistic since it may not be able to e¤ectively cluster data points with overlaps.

An interesting clustering approach using support vector concept is presented in [9], where data points are mapped to a high dimensional feature space and support vectors are used to de...ne a sphere to enclose them. Data points are clustered hierarchically by adjusting parameters in the kernel function that mathematically represents the mapping from input space to feature space; the outliers are allowed by setting appropriate penalty parameter. The method has advantages: ...nd clusters with arbitrary shapes, no need for dimensionality reduction and capability of dealing with outliers. However, the clusters with sizable overlaps cannot be correctly found by using this method, therefore it is not very suitable for microarray data clustering.

We propose a model-based approach hierarchical data exploration method that may greatly overcome the limitations of other methods. For instance, our method can evaluate the overall cluster structure while hierarchical clustering method and SOM can only do clustering blindly by starting without any idea about the large scope structure. The hierarchical visualization scheme help discover any hierarchical tree structure if existing, but it is still valid if such structure do not exist since the method is also designed for visualizing the inner structure of any cluster. The initialization of the clustering is also supported by user interaction and veri...ed by model selection criterion to ...nd the best structure description. The soft data decomposition allows the overlaps among clusters are well respected and modeled. The model selection procedure provide a theoretical and quantitative tool for cluster validation.

In this paper, we will report the progress in cluster discovery with our newly developed discriminatory data mining methods [16][17]. The presentation will entail three major components: (1) statistical modeling of gene expression microarray data with a standard ...nite normal mixture (SFNM) distribution; (2) development of a joint supervised and unsupervised data mining scheme to "discover" sample clusters in a discriminative visual pyramid; and (3) evaluation of the data clusters produced by such scheme with phenotype-known microarray experiments. Major di¤erences are found between our work and the previous most related research [18], [26], [22], [23], [24][25]. First, since the high complexity of the data structure within a high dimensional space cannot be adequately explored by a single-level visualization [18], we developed a hierarchical visualization paradigm, involving mixture statistical sub-models and visualization subspaces. The resulting data mining tool is capable of capturing cluster distribution structure in high dimensional space and discover the relationships among clusters. Second, we proposed three algorithms: 1) Discriminatory Component Analysis (DCA), 2) combined Projection Pursuit Method (PPM) / Independent Component Analysis (ICA) – ICA/PPM, and 3) combined PPM / Principal Component Analysis (PCA) – PCA/PPM. All three probabilistically project the softly partitioned data set onto multiple visual subspaces. They allow an e¤ective separation of local

clusters in dimensionally reduced visual subspaces, which may represent the original data set well. Third, we implemented a probabilistic adaptive principal components extraction (PAPEX) algorithm to estimate the top two principal axes and an incremental expectation-maximization (IEM) procedure to estimate SFNM distribution. The computation is e¢cient when confronted with high dimensional data sets [14]. Finally, we imposed a model selection procedure to determine the number of sub-clusters within each cluster using the Minimum Description Length (MDL) criterion. In addition, applying the MDL criterion also determines whether a further split or partition of a subspace should continue in completing the whole hierarchy [16], [24].

II. Theory

A. Hierarchical Visual Data Exploration Scheme

The purpose of cluster analysis is to determine whether there are certain number of well-de...ned data sets within the entire data distribution and/or derive most rational and optimal grouping scheme to partition data into a speci...ed number of clusters.

Since a gene expression microarray data set is a mixture of samples of cancer and non-cancer, or a mixture of samples of various types of cancers, the SFNM model may be the best approach for describing such multi-modal data structure [12]. In the case that k clusters exist in the data set, a mixture model with k normal distributions can be used to describe the overall distribution of the data. We will also estimate the density parameters of each cluster and the overall mixture.

Assume the sample points $\{t_i\}$ in gene expression space form K_0 clusters $\{(\mu_{t1}, C_{t1}), \ldots, (\mu_{tk}, C_{tk}), \ldots, (\mu_{tK_0}, C_{tK_0})\}$, where μ_{tk} and C_{tk} are the mean vector and covariance matrix of cluster k respectively. Using the SFNM to model the multi-modal distribution is considerably successful recently [12]. Such a data distribution takes a sum of the following general form

$$p(t) = \sum_{k=1}^{\infty} \pi_k g(t|\mu_{tk}, C_{tk}) \tag{1}$$

where π_k is the corresponding mixing proportion, with $0 \cdot \pi_k \cdot 1$ and $\sum \pi_k = 1$, and g is the

Gaussian kernel. The modeling of SFNM on the microarray data addresses a combination of the detection of structural parameter K_0 (e.g., cluster discovery) and the estimation of regional parameters (π_k, $^1_{tk}$,C_{tk}) based on the observations t. One natural criterion used for this modeling is the Maximum Likelihood (ML) estimation using the Expectation-Maximization (EM) algorithm [12].

The super high dimensionality (500 v 8000) of microarray data introduce di¢culties in the revelation of data structure, which have been well studied in [16]. Cover's theorem on the separability of patterns tells us that one single projection on a dimension reduced space is not su¢cient for revelation of the true data structure. Hierarchical visual exploration paradigm, involving hierarchical statistical models and visualization spaces/subspaces, may provide more opportunities for the user to understand the data distribution structure, and are essential for high dimensional microarray data study. We believe that the consideration of introducing user interaction into the clustering algorithm is a more practical approach, which greatly reduces both computational complexity and local optimum likelihood [16][11]. A user-friendly graphical interface for data visualization purpose is developed to allow the user to select initial centers of the data clusters. To visualize data, we further developed data projection methods based on the current methods used in [16] in order to maximize the revelation of cluster structure. The details about the various visualization techniques will be introduced in the next sub-section.

In this approach, the techniques involved are: statistical modeling of microarray data with SFNM distribution, discriminative data projections jointly presented by supervised and unsupervised learning processes, soft cluster decomposition based on an incremental expectation-maximization (IEM) procedure, and evaluation of the data clusters with phenotype-known microarray experiments.

The hierarchical version of the SFNM model can be extended to include more levels based on the same principle as above. The more hierarchical levels the tree has, the more sub-models are used, and the ...ner the sub-models are. The formation of the hierarchical visualization tree is guided and veri...ed by model selection over x-subspaces. Model selection refers to the detection of the structural parameter K (the number of clusters or sub-clusters). In addition to the user's visual inspection, we

propose to use an information theoretic criterion, i.e., the Minimum Description Length (MDL) [27]. The MDL calculation is a model ...tting procedure, in which an optimal model is selected such that the selected model best ...ts the observed data. Thus, the value of K is selected by minimizing

$$\mathrm{MDL}(K_a) = \; ¡ \log(L_{ML}) + 0.5 K_a \log N \tag{2}$$

where K_a is the number of free adjustable parameters, and L_{ML} is the joint maximum likelihood, of the model.

B. Discriminatory Data Projection

The purpose of developing discriminatory data projection tools is to maximally discover hidden cluster structure in the data space. The consideration of using multiple data projection tools is primarily based on the fact that the performance of the individual projection scheme tends to be case-dependent due to limited number of data samples in nearly all existing microarray data. Therefore, it is insu¢cient to use only one projection tool, which may increase risk of losing chances to discover cluster structure. The four discriminatory projection tools presented in this paper are: PCA, DCA, PCA/PPM, and ICA/PPM. The details of each method are discussed in the following sub-sections.

B.1 PCA

PCA is an e¤ective unsupervised method for achieving dimensionality reduction [22], [14], [10]. For a set of observed d-dimensional data vectors $\{t_i\}$, $i \in \{1, ..., N\}$, the q principal axes w_m, $m \in \{1, ..., q(\cdot d)\}$, are those orthogonal axes onto which the retained variances under projection are maximal. It can be shown that the principal axes w_m are given by the q dominant eigenvectors (i.e., q maximal eigenvalues) of the sample covariance matrix

$$C_t = \frac{1}{N} \sum_{i=1}^{N} (t_i ¡ {}^1{}_t)(t_i ¡ {}^1{}_t)^T \tag{3}$$

such that

$$C_t w_m = \lambda_m w_m \tag{4}$$

where ^1_t is the sample mean and λ_m is the eigenvalue. The vector $x_i = W^T(t_i - {}^1_t)$, where $W = (w_1, w_2, ..., w_q)$, is thus a q dimensional new representation of the observed vector t_i. Two issues contribute to the limitations of the conventional PCA: its global linearity without incorporating data structure; and its optimality based on reconstruction error rather than pattern separability.

B.2 DCA

If class information is known, the search of directions in data space for discovering cluster structure is under better guidance. There are two types of class information we may be able to obtain: known phenotypes from biological experimental setting, and sub-cluster information resulting from cluster decomposition based on an unsupervised projection (PCA or PPM). For the top level projection, DCA is a supervised process by using the known phenotype(class) information in the search of projection. However, DCA can also be used in an unsupervised situation that is on the sub-levels by using the second type of class information discussed above. Demonstrations of different applications of DCA are shown in the Result Section.

When confronting a multi-modal data set, however, is to emphasize the inter-cluster separation by replacing the total covariance matrix with the Fisher's scatter matrix [26], [10], i.e., to find the eigenvectors of $S_w^{-1} S_b$

$$S_w^{-1} S_b w_m = \lambda_m w_m \tag{5}$$

where the within-cluster scatter matrix (S_w) is the joint scatter of data point t_i around the conditional mean vector $^1_{tk}$ of K_D classes (on the top level) or sub-clusters (on the sub-levels)

$$S_w = \sum_{k=1}^{K_D} \pi_k C_{tk} \tag{6}$$

with cluster conditioned covariance matrix

$$C_{tk} = \frac{\sum_{i=1}^{N} z_{ik}(t_i - \mu_{tk})(t_i - \mu_{tk})^T}{\sum_{i=1}^{N} z_{ik}} \tag{7}$$

where

$$z_{ik} = \frac{\pi_k g(t_i | \mu_{tk}, C_{tk})}{p(t_i)}, \tag{8}$$

and the between-cluster scatter matrix (S_b) is the scatter of the cluster conditional mean vector μ_{tk} around the overall data center μ_t

$$S_b = \sum_{k=1}^{K} \pi_k (\mu_{tk} - \mu_t)(\mu_{tk} - \mu_t)^T \tag{9}$$

such that the separability of patterns is maximized, that is

$$W = \arg\max_{W_0} \{Trace(W_0^T S_w^{-1} S_b W_0)\}. \tag{10}$$

This is termed as Discriminatory Component Analysis.

The original vectors $\{t_i\}$ are linearly transformed by W, a $d \leq 2$ matrix, through $x = W^T(t - \mu_t)$ into a two-dimensional projection space $x = (x_1, x_2)^T$. For a normal distribution $g(t | \mu_{tk}, C_{tk})$ over the data space, a similar dimensionally reduced probability distribution $g(x | \mu_{tk}, C_{tk})$ of the new variable x in the projection space is simply defined by the Radon transform of $g(t | \mu_{tk}, C_{tk})$

$$g(x | \mu_{xk}, C_{xk}) = \int g(t | \mu_{tk}, C_{tk}) \delta(x - W^T t + W^T \mu_t) dt \tag{11}$$

where $\delta(.)$ is the delta function that $\delta(0) = 1$ and $\delta(\neq 0) = 0$. According to the linear superposition property of Radon transform and the projection invariant property of normal distribution, we have

$$f(x) = \sum_{k=1}^{K} \pi_k g(x | \mu_{xk}, C_{xk}) \tag{12}$$

as the counterpart of Eq. (1) in x-space defined by projection matrix W.

However, when the data set is projected onto a single lower dimensional subspace, its inherent multi-modal nature may be partially or completely obscured according to Cover's theorem on the separability

of patterns [14]. In other words, even though the cluster structure of a data set may be evident from the higher dimensional space, it is quite conceivable to have the ...ner cluster patterns concealed after a single linear projection, leading to an unidenti...able correspondence between Eq. (1) and Eq. (12) [16]. A novel approach is to model high-dimensional multi-modal data set with a hierarchical mixture model and accordingly with a collection of probabilistic principal discriminatory subspaces [16], [22], [23], [24], namely the exploratory cluster discovery.

Assume a top-level model consisting of a single Radon transform W and a mixture of $K_1 (< K_0)$ normal distributions $p(\mathbf{t}) = \sum_{k=1}^{K_1} \pi_k g(\mathbf{t}\,|\,\mu_{tk}, C_{tk})$ which is identi...able in x-space, i.e., $f(\mathbf{x}) = \sum_{k=1}^{K_1} \pi_k g(\mathbf{x}\,|\,\mu_{xk}, C_{xk})$, we can form a two-level hierarchy by associating a group of SFNM sub-models with each model k at top-level

$$p(\mathbf{t}) = \sum_{k=1}^{K_1} \alpha_k \sum_{j=1}^{K_{2,k}} \alpha_{j|k}\, g(\mathbf{t}\,|\,\mu_{t(k,j)}, C_{t(k,j)}) \tag{13}$$

where $\pi_{j|k}$ again corresponds to a set of mixing proportions, one for each k, with $0 \le \pi_{j|k} \le 1$ and $\sum_j \pi_{j|k} = 1$, and $\sum_{k=1}^{K_1} K_{2,k} = K_0$. To reveal the hidden cluster pattern within each model k at top-level, i.e., $g(\mathbf{t}\,|\,\mu_{tk}, C_{tk}) = \sum_{j=1}^{K_{2,k}} \pi_{j|k} g(\mathbf{t}\,|\,\mu_{t(k,j)}, C_{t(k,j)})$, an associated probabilistic principal discriminative subspace is constructed that focuses on the separability of patterns within the data portion de...ned by model k, where the opaque degree of a data point in the subspace plot is proportional to its posterior probability belonging to this model, i.e., z_{ik} determined at top-level.

The further cluster discovery is a two-stage procedure: a soft partitioning of each model k into $K_{2,k}$ sub-clusters followed by a construction of corresponding subspace. Instead of assigning each given data point exclusively to one subspace, the contribution to its generation is shared among all the subspaces. The subspaces for the sub-models at second-level are generated by the probabilistic DCA such that

$$S_{k,w}^{-1} S_{k,b} w_{k,m} = \lambda_{k,m} w_{k,m} \tag{14}$$

where $S_{k,w} = \sum_{j=1}^{K_{2,k}} \pi_{j|k} C_{t(k,j)}$ with subcluster conditioned covariance matrix

$$C_{t(k,j)} = \sum_{i=1}^{N} z_{i(k,j)} (\mathbf{t}_i - \mu_{t(k,j)})(\mathbf{t}_i - \mu_{t(k,j)})^T / \sum_{i=1}^{N} z_{i(k,j)},$$

$z_{i(k,j)} = z_{ik}\pi_{jjk}g(t_j\,|\,\mu_{t(k,j)},C_{t(k,j)})/g(t_j\,|\,\mu_{tk},C_{tk})$,

and $S_{k,b} = \sum_{j=1}^{K_{2,k}} \pi_{jjk}(\mu_{t(k,j)} - \mu_{tk})(\mu_{t(k,j)} - \mu_{tk})^T$. The probability distribution of model k in x-space at second-level is now de...ned by the model k focused Radon transform of $g(t_j\,|\,\mu_{tk},C_{tk})$, i.e., $g(x_j\,|\,\mu_{xk},C_{xk}) = \int_R g(t_j\,|\,\mu_{tk},C_{tk})\delta(x - W_k^T t + W_k^T\mu_{tk})dt$. It should be noted that each component in Eq. (13) now corresponds to an independent sub-model with projection matrix W_k. To interpret the corresponding set of visualization subspaces, all data points $x_{ik} = W_k^T(t_i - \mu_{tk})$ will appear in every plot of the K_1 subspaces at the second-level, with their opaque degree proportional to z_{ik}.

B.3 PCA/PPM

In the e¤ort of searching projections for cluster separability, we take an alternative unsupervised approach, Projection Pursuit Method (PPM) that is to search "interesting" projections. Even though it is not universally agreed on what constitutes an "interesting" projection in PPM research community, the de...nition of "interesting" projection by some leading researchers does meet our needs in this particular project, which is a projection where the data separate into distinct and meaningful clusters [20]. We have two particular goals of using PPM in this project: (1) ...nd low dimensional (equal or less than three) projections that provide the most revealing view of the overall data distribution; (2) use PPM for dimensionality reduction so that we will focus directly on the discriminatory projections rather than indirectly searching through covariance. The advantage of PPM is that it ...nds the directions that are not a¤ected by the linear scale and correlational structure of the data, which is the disadvantage of PCA.

As mentioned before if we want to put human ability for pattern discovery to good use of four or higher dimensional data, we shall ...rst look at the projections onto the spaces spanned by two or three of the dimensions. In most cases, any arbitrary direction could be the right one for cluster structure discovery. It implies that we have to search the space in all possible directions, thus the problem gets even worse due to exhaustive searching. In our approach, we tried to simplify the PPM by using non-

Gaussianity as a criterion, and using PCA and Independent Component Analysis (ICA) as vehicles for ...nding discriminatory projections.

The direction with Gaussian or super-Gaussian distribution is the one consisting least data structural information; on the other hand, the least Gaussian distribution indicates plentiful structural information. If the data distribute as one Gaussian or super-Gaussian distribution (a "spiky" probability density function (pdf) with long and heavy tails) in a direction, it implies that the data points are most likely forming a one-cluster structure instead of multi-modal structure that is the important for cluster separation. On the contrary, the data may construct two or more clusters in the directions where distributions are non-Gaussian, e,g. sub-Gaussian (a "‡at" pdf and more like a uniform distribution). We used one of the non-Gaussianity measures kurtosis, de...ned as $E\,fy^4g\ ¡\ 3\,(E\,fy^2g)^2$, where y denotes the random variable. Kurtosis can be either positive or negative. A random variable with a positive kurtosis is considered as a super-Gaussian, and that with a negative kurtosis is considered as a sub-Gaussian [21][15].

We try to fully utilize our PCA results to test PPM in order to reduce computational load. A prototype computer algorithm, termed as PCA/PPM, is implemented to calculate kurtosis of each principal component resulting from PCA and rank them so that the optimal directions are found to be those that show the strongest sub-Gaussian.

B.4 ICA/PPM

Independent component analysis (ICA) is a recently developed method for ...nding linear representations of non-Gaussian data such that the components are statistically independent, or as independent as possible. Since PPM is designed to search the directions with the least Gaussian distribution and the least Gaussian distribution is the criterion to estimate ICA model, ICA and PPM are closely related. The non-Gaussianity measures can be adopted as projection pursuit functional indices. ICA/PPM algorithm that is PPM assisted with ICA, is then implemented to directly search for directions with

non-Gaussian distribution in data space [10] [21]. Similar to PCA/PPM, kurtosis is calculated on each independent component resulting from ICA, and two components with the most negative kurtosis are chosen for 2-D projection.

III. Algorithm

We now present the description of our algorithms that progressively proceeds by ...tting a series of sub-models to the clusters of the data set interactively and incrementally.

The algorithm begins by determining W for the top-level projection (a two-dimensional x-space). The initial estimate of W is obtained from our previously developed APEX neural computation (e.g., Eq. (4)) [14], and further modi...ed by DCA-APEX algorithm (e.g., Eq. (5) with or without S_w^{-1}) [16] where the prerequisite is to estimate the SFNM model at top-level (e.g., Eq. (1)). To remedy the problem of high dimensionality with microarray data, neural computation of (W,¼$_k$, $^1{}_{tk}$,C_{tk}) is e¢cient, in which only the top two eigenvectors of the covariance or scatter matrix are calculated, and model parameter values are ...rst estimated in x-space and then ...ne tuned in t-space incrementally. For example, the Incremental EM (IEM) procedure provides "soft" splits of the data points, hence allowing the data to contribute simultaneously to multiple clusters, which results in

E-Step

$$z_{(i+1)k} = \frac{\pi_k^{(i)} g(x_{i+1}\mid {}^1{}_{xk}^{(i)}, C_{xk}^{(i)})}{f(x_{i+1}\mid ¼_k^{(i)}, {}^1{}_{xk}^{(i)}, C_{xk}^{(i)})}, \tag{15}$$

M-Step

$${}^1{}_{xk}^{(i+1)} = {}^1{}_{xk}^{(i)} + a(i)(x_{i+1} - {}^1{}_{xk}^{(i)})z_{(i+1)k}, \tag{16}$$

$$C_{xk}^{(i+1)} = C_{xk}^{(i)} + b(i)[(x_{i+1} - {}^1{}_{xk}^{(i)})(x_{i+1} - {}^1{}_{xk}^{(i)})^T - C_{xk}^{(i)}]z_{(i+1)k}, \tag{17}$$

$$\pi_k^{(i+1)} = \frac{i}{i+1}\pi_k^{(i)} + \frac{1}{i+1}z_{(i+1)k} \tag{18}$$

for $k = 1, ..., K_1$, where $a(i)$ and $b(i)$ are introduced as the learning rates, two sequences converging to zero, ensuring unbiased estimates after convergence. The user will pin-point the initial cluster centers

$^{1}{}^{(0)}_{xk}$ and assign $\pi_k^{(0)} = 1/K_1$ and $C_{xk}^{(0)} = W^T C_t W$. The optimum value of K_1 is determined based on MDL (e.g., Eq. (2)) where $K_a = 6K_1$ ¡ 1.

Determination of the subspaces W_k and sub-models $(\pi_{j|k}, {}^{1}{}_{t(k,j)}, C_{t(k,j)})$ at the second-level can again be viewed as a two-step estimation problem, in which further splitting of the sub-models is determined within each of the clusters identi...ed at the top-level such that its internal structures can be further explored over cluster-focused x-space. The initial estimate of W_k can be obtained using a probabilistic APEX (PAPEX) algorithm.

The corresponding $\sum_{j=1}^{K_{2,k}} \pi_{j|k} g(t_j; {}^{1}{}_{t(k,j)}, C_{t(k,j)})$ can again be estimated using IEM algorithm to allow a SFNM distribution with $K_{2,k}$ sub-models to be ...tted to cluster k, where the user will pin-point the initial subcluster centers ${}^{1}{}^{(0)}_{x(k,j)}$ and assign $\pi_{j|k}^{(0)} = 1/K_{2,k}$ and $C_{x(k,j)}^{(0)} = W_k^T C_{tk} W_k$ to initialize $\sum_{j=1}^{K_{2,k}} \pi_{j|k} g(x_j; {}^{1}{}_{x(k,j)}, C_{x(k,j)})$ with a model selection procedure. By replacing $z_{ik}(t_i ; {}^{1}{}_{tk})$ in PAPEX formulation with $\pi_{j|k}({}^{1}{}_{t(k,j)} ; {}^{1}{}_{tk})$, W_k is updated by a DCA-PAPEX procedure to generate a separability-based and cluster-focused subspace for model k at the second-level.

The construction of the entire hierarchical tree is completed when no further data splitting is recommended in all of the parent subspaces, followed by the generation of the bottom-level subspaces (for example, the third-level). The value of $W_{(k,j)}$ is obtained using the PAPEX algorithm with $z_{i(k,j)}$ instead of z_{ik}, and all data points $x_{i(k,j)} = W_{k,j}^T (t_i ; {}^{1}{}_{t(k,j)})$ will appear in every plot of the total K_0 subspaces at the bottom-level, with their opaque degree equal or proportional to $z_{i(k,j)}$.

IV. Results

A demonstration of the capability of ...nding cluster structure by PCA/PPM, DCA, and PPM is ...rst done on a simulated data set that consists of three dimensions and four clusters (N=100 for each cluster). The results are illustrated in Figure 1, where we can see that the maximal information about the cluster structure is revealed by using DCA, PCA/PPM, and ICA/PPM comparing to the conventional PCA. The four cluster structure is clearly shown in (b), (c), and (d) in Figure 1, but only

three clusters are seen in the PCA plot (a) without incorporating color information in all plots. We shall make it clear that PCA, PCA/PPM and ICA/PPM are all completely unsupervised processes without replying on the known class information, except DCA that is supervised in the case. The class information is used only to show the four distinct classes with four di¤erent colors and symbols.

The model selection of simulated data at the top level projection generated by DCA is performed, and the results showed that a four-cluster structure may best describe the data distribution on this level. In the Figure 2, ...ve di¤erent model selection patterns are tested and MDL curve is plotted, and the MDL suggests that the four-cluster structure is best.

A hierarchical visualization trees, as shown in Figure 3(a), is generated on the simulated data set. The top ...gure is a top level projection of the complete data set, where we can only see three clusters, the middle ...gure is a second level projection that provides individual di¤erent views of the three sub-clusters selected in the top level projection. In the second level, we can see two hidden clusters in sub-cluster #1, this gives the user opportunities to discover more information about data structure, and it makes further partitioning possible. Clusters are partitioned and shown in their own windows in the bottom ...gure. In this experiment, only conventional PCA is used to generate all projections.

To illustrate a joint way of the discovery data structure by combining PCA, DCA, PCA/PPM and ICA/PPM, we also tested PCA/PPM, DCA, and ICA/PPM in the generation of hierarchical visualization tree. In this case, since conventional PCA is unable to locate the directions in the space where real cluster structure can be displayed, we can use PCA/PPM, DCA, and ICA/PPM as alternatives. When DCA is used to plot the top level projection in Figure 3(b), more information about the cluster structure is revealed after the ...rst step, i.e., the directions to show the four-cluster structure are found in the space by DCA. For the simulated data set, the hierarchical visualization by DCA is used as an illustration because DCA, PCA/PPM and ICA/PPM are similar to one another. The two hierarchical trees in Figure 3 are produced independently. The consistency of the clustering results and the known class grouping can be seen from the color of the data points in each individual

window on the bottom level. The fact that the data points within one cluster have the same color implies that the data points belonging to the same phenotype group are grouped into one cluster.

Besides testing the methodology on the simulated data, we also evaluate the real microarray data sets from National Cancer Institute (NCI) and Massachusetts Institute of Technology (MIT). In the 2308 dimensional (genes) microarray data sets of round blue cell tumors from NCI, there are four classes: neuroblastoma (NB; N=12), rhabdomyosarcoma (RMS; N=21), non-Hodgkin lymphoma (NHL; N=8) and the Ewing family of tumors (EWS; N=23). Figure 4 illustrates how all projection methods (PCA, PCA/PPM, DCA, and ICA/PPM) on NCI data explore cluster structure. According to our experience, these four methods may have some extent on ...nding direction for discovering cluster structure in various cases. Thus, using all applications to examine the data structure are more informative than one or two methods combined. As discussed above, in this experiment, the known phenotype information has been only used for color ...gure plotting in PCA, PCA/PPM and ICA/PPM experiments rather than in projection searching, except for DCA experiment that is supervised.

PCA and PCA/PPM on the NCI data generate hierarchical visualization trees (Figure 5). Using PCA to generate 2-D projections produces the left hierarchical tree Figure 5(a), and applying the PCA/PPM generates the right tree Figure 5(b). MDL curves are also plotted for the two di¤erent top level projections. The curves indicate that the three-cluster from PCA and the four-cluster structures from PCA/PPM are best in describing the data distribution at the top level projections. The number of clusters determined by MDL agrees with the user visual inspection. As discussed in the hierarchical exploration experiment on the simulated data, the consistency of the clustering and the known biological phenotypes de...ned above is shown by the uni...ed color of data points within each cluster. The clustering scheme recommended by MDL measure also indicates the consistency of the biological phenotype information and the ...nal clustering in the bottom level of Figure 5. In Figure 5 (a), although MDL cannot catch the four-cluster structure on the top level, the projections on the second level do provide good opportunity for discovering two clusters within the sub-cluster

#1 (mixed green and yellow) on the top level. This also demonstrates the advantages of hierarchical visual exploration scheme on the cluster discovery. MDL recommends a four cluster structure on the top level of Figure 5(b), the well partitioned four clusters on the bottom level show the ability of MDL to the ...nding the true cluster structure.

In studying leukemia, MIT published a 7129 dimensional microarray data sets that contain two classes, acute lymphoblastic leukemia (ALL; N=47) and acute myeloid leukemia (AML; N=25). The 2-D projections of the MIT data are presented in Figure 6 . All four projections on the leukemia data sets explore the data structure similarly to the other cases (simulated and NCI data sets).

DCA is meaningful with the model selection and the cluster partitioning even under unsupervised condition, i.e. no class information is known. We demonstrate this idea by combining PCA and DCA dynamically at the top level projection. In Figure 7, the top level projection in the top ...gure is generated by PCA (unsupervised analysis) without knowing any class information. After model selection and partitioning (provide class information), DCA (supervised analysis) can now produce the re-projection of the data (middle ...gure). The bottom ...gure is a partition of the re-projected data. Even though the PCA projection (top ...gure) can be directly partitioned into sub-clusters (bottom ...gure), the additional step using DCA (middle ...gure) provides another chance to visualize the complete data set from di¤erent angles in which cluster structure is emphasized. As in this example, this data projection scenario is especially useful when cluster structure is ambiguous to the user in one projection, but becomes clear after DCA is applied based on the user model selection and cluster partitioning. From the clustering results shown on the bottom level of Figure 7, we can conclude that the clustering is fairly consistent with the biological phenotype information since only a few mixed groupings occur.

V. Discussion

The revelation of growing volume of high dimensional and multi-modal data demands a data mining tool di¤ering from conventional data visualization method, which is capable of dealing with high dimensional data set. The hierarchical visualization paradigm involving hierarchical statistical models and visualization space is proven to be able to e¤ectively discover data structure and capture all interesting aspects of the data. Using several complementary visualization subspaces makes this complicated task feasible. The strategy of the hierarchical data exploration and mining tool used for cluster discovery is that the top level model and projection should explain the whole structure of the data set, while lower level models explain the local and internal structure between individual cluster, which may not be obvious in the high level models. With several complementary mixture models and visualization projections, each level will be relatively simple while the complete hierarchy maintains overall ‡exibility yet still conveys considerable cluster information. In this algorithm, dimensionality reduction and cluster decomposition are two major components. The dimensionality reduction allows visualization of high dimensional data and less computational demand. The cluster decomposition provides relatively simple models by partitioning large and complicated mixture models into small local structure, which o¤ers great ease of interpretation and many bene...ts of analytical and computational simpli...cation.

The techniques involved are statistical modeling of the high dimensional data with SFNM distribution, 2-D data projection jointly presented by unsupervised and supervised data mining scheme, and evaluation of cluster structure produced by such scheme using microarray experiments with known phenotypes. Di¤erent from conventional PCA, the PCA/PPM, DCA and ICA/PPM project entire data set and the softly partitioned sub-clusters onto multiple 2-D visual subspaces, which makes every subcluster/subspace be discriminatively explored individually so that local cluster structure is e¤ectively revealed. Furthermore, the PAPEX and incremental expectation-maximization (IEM) procedure are implemented to estimate SFNM distribution. With the model-based approach, a model selection procedure to determine the number of sub-clusters within each cluster using the minimum description

length criterion. This allows algorithm to automatically determine whether a further split of a subspace should continue in completing the whole hierarchy [16]. User interaction with the algorithm is also an important issue. The user-friendly graphical interface facilitates the data visualization purpose, which allows the user to select initial centers of the data clusters. Our experience has convincingly indicated a great reduction of both computational complexity and local optimum likelihood. Although the ...nal SFNM model can be estimated, the pathways of achieving cluster decomposition may be multiple. This user-driven nature of the current algorithm is also highly appropriate for the visualization context. With such features in the data mining algorithm, our tools can explore data structure in great extents with no standard data analyses may compare.

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VI. List of Figures

1. The 2-D projections of the simulation data resulting from conventional PCA, PCA/PPM, DCA, and ICA/PPM.

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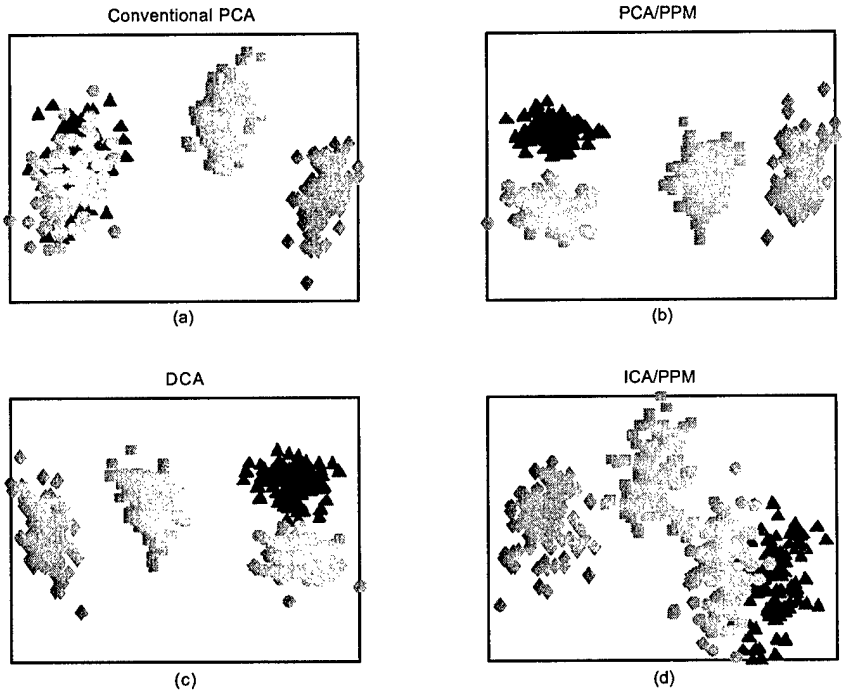

Fig. 1. The 2-D projections of the simulation data resulting from conventional PCA, PCA/PPM, DCA, and ICA/PPM.

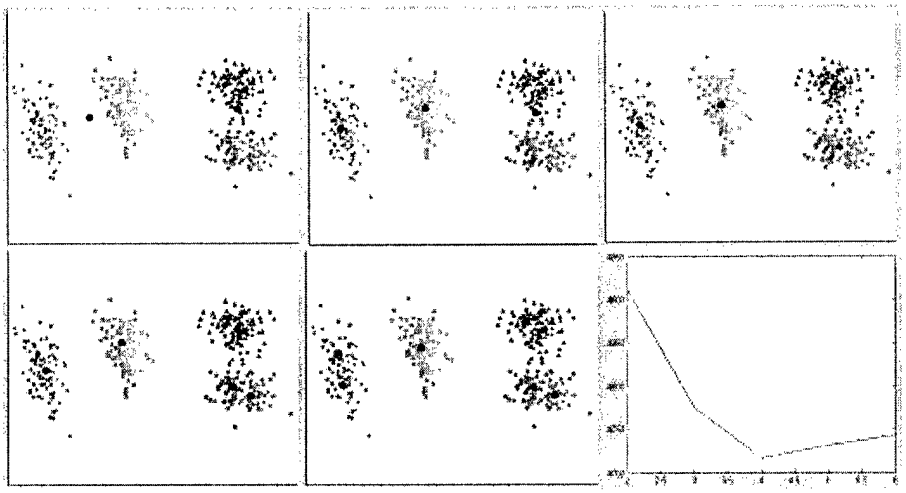

Fig. 2. The model selection and the MDL curve of the simulated data at the top level projection.

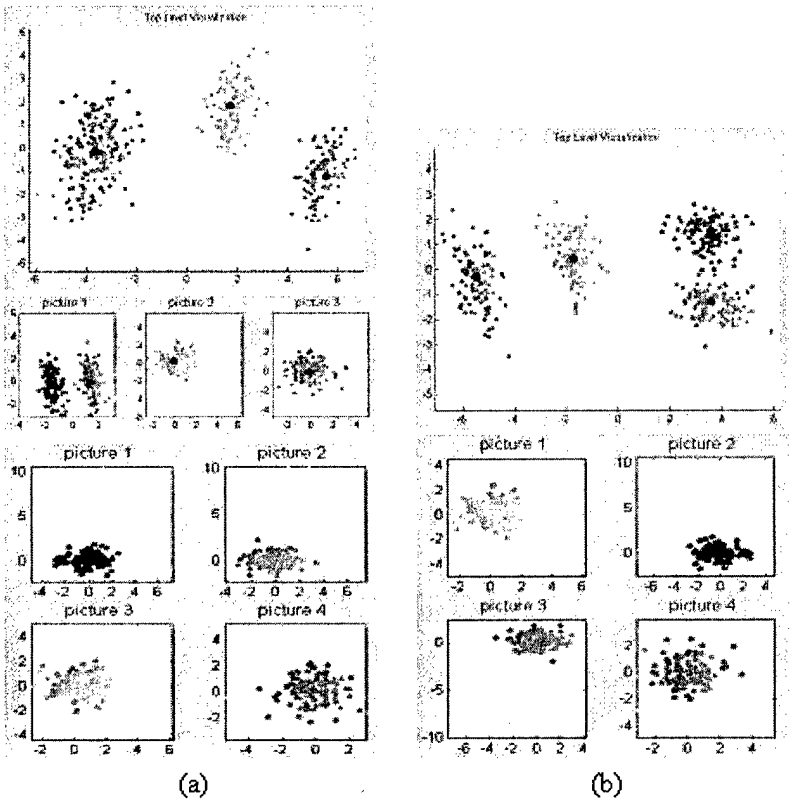

Fig. 3. The hierarchical visualization trees of the simulated data. The left ...gure (a) is generated by using PCA, and right ...gure (b) are generated by using DCA.

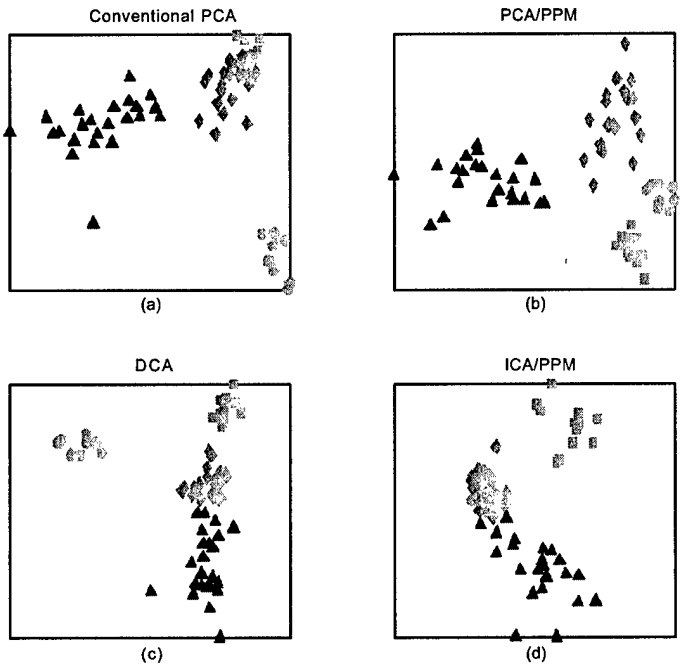

Fig. 4. The 2-D Projections of the NCI data resulting from PCA, PCA/PPM, DCA, and ICA/PPM.

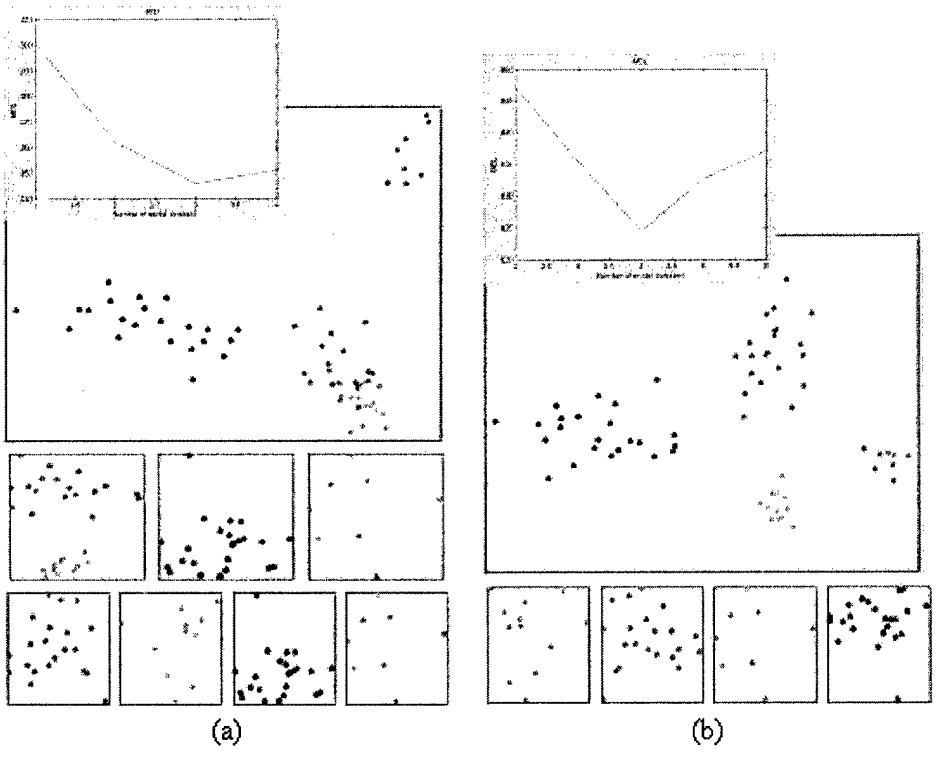

Fig. 5. The hierarchical visualization trees of NCI data. The left ...gure (a) is generated by using PCA, and right ...gure (b) is generated by using PCA/PPM. MDL curves for the top level projections are also included.

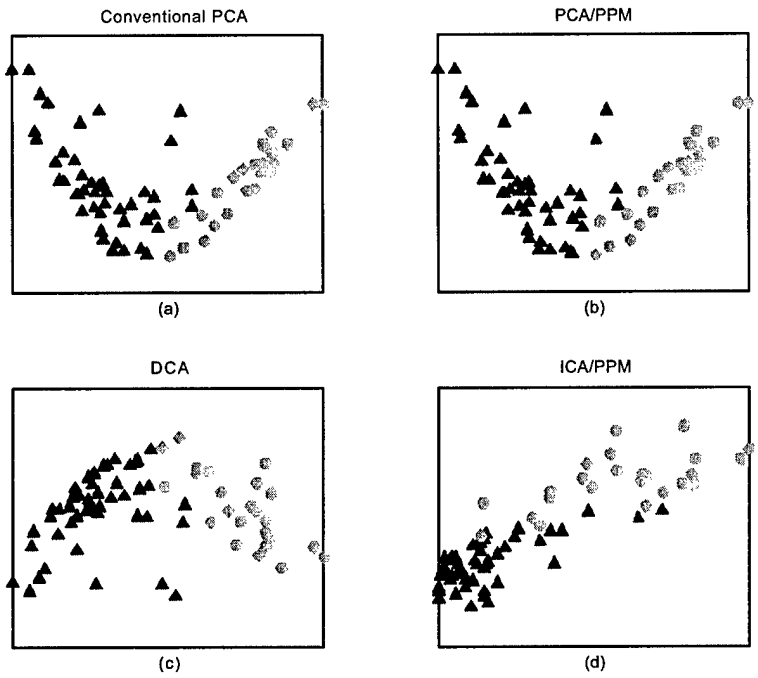

Fig. 6. The 2-D Projections of the MIT data resulting from PCA, PCA/PPM, DCA, and ICA/PPM.

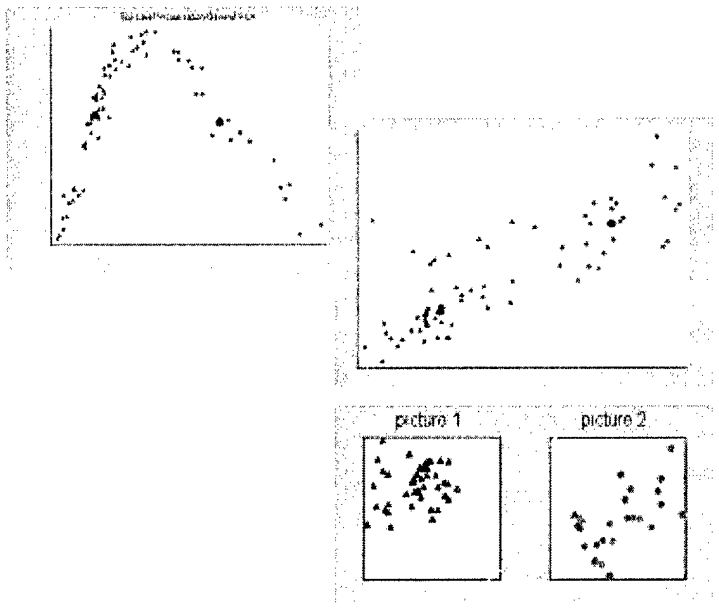

Fig. 7. The 2-D Projections of the MIT data resulting from PCA, PCA/PPM, DCA, and ICA/PPM.

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Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling

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Antiestrogens include agents such as tamoxifen, toremifene, raloxifene, and fulvestrant. Currently, tamoxifen is the only drug approved for use in breast cancer chemoprevention, and it remains the treatment of choice for most women with hormone receptor positive, invasive breast carcinoma. While antiestrogens have been available since the early 1970s, we still do not fully understand their mechanisms of action and resistance. Essentially, two forms of antiestrogen resistance occur: *de novo* resistance and acquired resistance. Absence of estrogen receptor (ER) expression is the most common *de novo* resistance mechanism, whereas a complete loss of ER expression is not common in acquired resistance. Antiestrogen unresponsiveness appears to be the major acquired resistance phenotype, with a switch to an antiestrogen-stimulated growth being a minor phenotype. Since antiestrogens compete with estrogens for binding to ER, clinical response to antiestrogens may be affected by exogenous estrogenic exposures. Such exposures include estrogenic hormone replacement therapies and dietary and environmental exposures that directly or indirectly increase a tumor's estrogenic environment. Whether antiestrogen resistance can be conferred by a switch from predominantly ERα to ERβ expression remains unanswered, but predicting response to antiestrogen therapy requires only measurement of ERα expression. The role of altered receptor coactivator or corepressor expression in antiestrogen resistance also is unclear, and understanding their roles may be confounded by their ubiquitous expression and functional redundancy. We have proposed a gene network approach to exploring the mechanistic aspects of antiestrogen resistance. Using transcriptome and proteome analyses, we have begun to identify candidate genes that comprise one component of a larger, putative gene network. These candidate genes include NFκB, interferon regulatory factor-1, nucleophosmin, and the X-box binding protein-1. The network also may involve signaling through ras and MAPK, implicating crosstalk with growth factors and cytokines. Ultimately, signaling affects the expression/function of the proliferation and/or apoptotic machineries.
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Introduction

Antiestrogens primarily act by competing with estrogens for binding to the estrogen receptor (ER) and are the most widely administered endocrine agents for the management of ER-expressing breast cancers. The first antiestrogens were generated in the mid-1950s as fertility agents and included ethamoxytriphetol (MER-25) and clomiphene. The ability of these compounds to induce responses in some breast cancer patients soon became apparent (Kistner and Smith, 1960), but the compounds induced significant toxicity (Herbst *et al.*, 1964). In the early 1970s, the first study in breast cancer patients was published with a new antiestrogen tamoxifen (TAM, ICI 46474) (Cole *et al.*, 1971). Over the next 17 years, the total exposure to TAM reached 1.5 million patient years (Litherland and Jackson, 1988) and other selective estrogen receptor modulators (SERMs) are being developed and studied. TAM is now the most frequently prescribed antiestrogen, and compelling data have demonstrated a significant overall survival benefit with the administration of this agent in breast cancer patients with endocrine responsive disease (EBCTCG, 1992, 1998).

When compared with cytotoxic chemotherapy, antiestrogens are well tolerated and are associated with mostly minor toxicities (Love, 1989). Common side effects associated with TAM therapy include vasomotor symptoms, gastrointestinal disturbance, atrophic vaginitis, and changes in sexual functioning (Day *et al.*, 1999). While the frequency and severity of hot flashes and other toxicities can be particularly unpleasant for some women, remarkably few discontinue TAM because of these side effects. Medical indications for the

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prompt discontinuation of therapy include associated venous thromboembolic disease and endometrial cancer (typically invasive adenocarcinoma, although uterine sarcomas have been reported). The incidence of these events is very low, and screening methods for both deep vein thrombosis and endometrial abnormalities exist. However, these increased risks must be considered in the light of the potential benefits—particularly in the case of healthy women considering TAM in the setting of chemoprevention as opposed to active treatment. The development of both venous thromboembolic disease and endometrial cancer is attributed to the estrogenic effects of TAM and may be abrogated by the development of more SERMs (e.g., raloxifene) or of pure ER antagonists (e.g., ICI 182,780; fulvestrant) (Robertson, 2001).

Some antiestrogens produce beneficial effects beyond their ability to inhibit existing breast cancers. The most convincing evidence supports an association between TAM treatment and a marked reduction in the risk of developing a contralateral breast cancer (EBCTCG, 1992) and a significant reduction in the incidence and severity of osteoporosis in postmenopausal women (Freedman *et al*., 2001; Kinsinger *et al*., 2002). Several early studies suggested a reduction in the risk of cardiovascular disease with TAM therapy, but this is not consistently reported (EBCTCG, 1998; Fisher *et al*., 1998). When observed, the cardiovascular benefit was usually attributed to the estrogenic effects of TAM; both estrogens and TAM produce apparently beneficial changes in serum triglyceride and cholesterol concentrations (Joensuu *et al*., 2000), perhaps through effects mediated by apolipoprotein E (Liberopoulos *et al*., 2002). However, these findings must be considered in the light of recent large studies of estrogenic hormone replacement therapy (HRT) that either failed to identify an HRT-induced reduction in coronary heart disease (Hulley *et al*., 1998; Grady *et al*., 2002; WHI, 2002) and stroke (Viscoli *et al*., 2001; WHI, 2002), or demonstrated an increase in the risk of these diseases.

An overview of antiestrogen resistance

Despite the relative safety and significant antineoplastic and chemopreventive activities of antiestrogens, most initially responsive breast tumors acquire resistance (Clarke *et al*., 2001b). It is unlikely that any single mechanism or single gene confers antiestrogen resistance. Rather, several mechanisms likely exist that encompass pharmacologic, immunological, and molecular events. These mechanisms, none of which are fully understood, likely vary within tumors. Intratumor variability in antiestrogen responsiveness will reflect the presence of multiple cell subpopulations (Clarke *et al*., 1990a). Since breast cancers appear highly plastic and adaptable to selective pressures, the intratumor diversity in antiestrogen responsive subpopulations also likely changes over time. Tumors appear capable of dynamically remodeling their cell populations in response to changes in host immunity or endocrinology, or the administration of local or systemic therapies. This plasticity is probably both cellular (some existing populations die out/back while other populations become dominant) and molecular (new cell populations emerge as individual cells/populations adapt their phenotypes by modifying their transcriptomes/proteomes).

Since the major pharmacologic and immunologic mechanisms of antiestrogen resistance have been previously reviewed (Clarke *et al*., 2001b), we will focus on the role of molecular signaling through ER-mediated activities in antiestrogen responsiveness. Antiestrogen resistance can be either *de novo* or acquired. The most common and best defined mechanism of *de novo* resistance is the absence of both ER and progesterone receptor (PR) expressions. However, we fail to predict response to antiestrogens in approximately 25% of ER+/PR+, 66% of ER+/PR−, and 55% of ER−/PR+ breast tumors (Honig, 1996). Many ER+ and/or PR+ breast tumors are already resistant by the time of diagnosis and the resistance mechanism in these tumors is unknown.

Overall, a loss of antiestrogen responsiveness by initially responsive tumors is likely to be the most common acquired resistance phenotype. Most initially antiestrogen responsive tumors retain levels of ER expression at recurrence on antiestrogen therapy that would still define them as being ER+ (Encarnacion *et al*., 1993; Kuukasjarvi *et al*., 1996; Bachleitner-Hofmann *et al*., 2002). Most data are for TAM treatment; ICI 182780, which causes degradation of ER (Dauvois *et al*., 1992), may have a greater potential for producing ER− tumors (Kuukasjarvi *et al*., 1996). From our *in vitro* studies, loss of ER is not required to achieve resistance to either ICI 182,780 or TAM (Brünner *et al*., 1993b, 1997). The loss of ER expression upon recurrence despite adjuvant TAM therapy has been reported in less than 25% of tumors (Kuukasjarvi *et al*., 1996; Bachleitner-Hofmann *et al*., 2002). Overall, a loss of ER expression does not seem to be the major mechanism driving acquired antiestrogen resistance.

A different resistance phenotype has been described in human breast cancer xenografts that exhibit a switch to a TAM-stimulated phenotype. This mechanism of clinical but not pharmacologic resistance may not be the dominant antiestrogen resistance phenotype. If the prevalence of acquired resistance phenotypes in ER+ tumors broadly reflects what is seen in *de novo* resistance, then the dominant resistance phenotype is a loss of antiestrogen responsiveness.

Whether the continued expression of ER is required for antiestrogen-resistant tumor growth or survival is not known. However, responses to aromatase inhibitors after an initial response and then failure on TAM are common (Buzdar and Howell, 2001) and strongly suggest that some TAM-resistant tumors retain a degree of estrogen responsiveness. Where durations of responses to second-line endocrine manipulations are short, truly estrogen-independent cell populations are either already present at the time of recurrence and/or many cells in the tumor are able to adapt rapidly to further changes in their endocrine environment. Very

short response durations or disease stabilization may reflect the withdrawal of a mitogenic stimulus that is not required for the survival or basal proliferation of most cells in the tumor.

Antiestrogens

TAM is a triphenylethylene and its triaryl structure has been widely copied in the design of new compounds. Several TAM derivatives are already available, including toremifene (chloro-tamoxifen) and droloxifene (3-hydroxytamoxifen). Not surprisingly, both drugs are essentially equivalent to TAM in terms of their antitumor activities and toxicities (Roos *et al.*, 1983; Pyrhonen *et al.*, 1999), so neither is widely used in clinical practice.

The characteristic of raloxifene that has attracted the most interest is its apparent lack of estrogenic effects in the uterus, resulting in great interest in this drug's potential role in breast cancer chemoprevention. Subgroup analysis of the data from the Multiple Outcomes of Raloxifene (MORE) trial revealed that administration of raloxifene was associated with a 75% reduction in the incidence of invasive breast cancer without a concurrent increase in the incidence of endometrial cancers (Cummings *et al.*, 1999). This finding has led to the ongoing randomized study of TAM and raloxifene (STAR) in breast cancer prevention. Raloxifene still acts as an antiestrogen in the brain, increasing the incidence of hot flashes (Davies *et al.*, 1999). A high incidence of severe hot flashes is problematic for a drug to be administered for approximately 5 years to otherwise apparently healthy women. Raloxifene was recently approved by the Food and Drug Administration for the treatment and prevention of osteoporosis in postmenopausal women. While a benzothiophene, raloxifene (keoxifene; LY 156,758) has a three-dimensional structure broadly similar to the triphenylethylenes.

ICI 182,780 (Faslodex; Fulvestrant) is among the more promising new antiestrogens. Unlike TAM, ICI 182,780 is a steroidal ER inhibitor that is often described as a 'pure' antagonist with no estrogenic activity. This is in comparison to the triphenylethylene and benzothiophene antiestrogens, which are nonsteroidal, competitive ER inhibitors with partial agonist activity. The pure antagonist is characterized by antineoplastic activity in breast cancer and is devoid of uterotropic effects. However, the lack of agonist activity limits beneficial effects in bone. Whether ICI 182,780 also will increase hot flashes depends on whether it reaches adequate concentrations in the brain. Unlike TAM (Clarke *et al.*, 1992), ICI 182,780 appears to be a substrate for the P-glycoprotein efflux pump (De Vincenzo *et al.*, 1996), a major contributor to the blood–brain barrier (Cordon-Cardo *et al.*, 1989). Consistent with this observation, initial studies suggest that this antiestrogen does not enter the brain in high concentrations (Howell *et al.*, 1996). Pure antagonists may further exacerbate bone loss, a concern that also applies to aromatase inhibitors (Dowsett, 1997), but this issue may be addressed with the concurrent use of bisphosphonates or other therapies for osteoporosis. Clinical experience with ICI 182,780 has been reviewed by Howell (2001).

Antiestrogens and breast cancer treatment

Antiestrogens are effective in the adjuvant, metastatic, and chemopreventive settings and clearly induce significant increases in overall survival in some breast cancer patients (EBCTCG, 1992, 1998). Unlike aromatase inhibitors (inhibit estradiol biosynthesis), which are administered as single agents only to women with nonfunctioning ovaries, TAM can be given irrespective of menopausal status. In the adjuvant setting, TAM is administered at a daily oral dose of 20 mg, and several studies have now shown that the optimal duration of treatment is 5 years. While shorter (2 years) and longer (10 years) treatment durations produce notable responses, the risk : benefit ratios are strongly in favor of 5 years of treatment (Stewart *et al.*, 1996; EBCTCG, 1998).

While molecular predictors of tumor responsiveness are rare for most breast cancer treatments, expressions of ER and PR strongly predict for a response to antiestrogens. Up to 75% of breast tumors expressing both receptors (ER+/PR+) respond to TAM. Response rates are somewhat lower in ER+/PR− tumors (~34%) and ER−/PR+ tumors (45%). The response rate in ER−/PR+ may be an overestimate; relatively few tumors with this phenotype have been evaluated and the ER− assessment may include false-negative ER measurements. Only a small proportion of ER−/PR− tumors respond to antiestrogens (<10%), perhaps also reflecting false-negative ER measurements. Indeed, the most recent meta-analysis from the Early Breast Cancer Trialists Collaborative Group (EBCTCG) found no significant reduction in recurrence rates in patients with ER-poor tumors who received adjuvant TAM (EBCTCG, 1998).

Results of the 1998 EBCTCG meta-analysis found limited evidence for a TAM-induced increase in the risk of death from any cause in women with ER-poor tumors. Why TAM might be detrimental to some women is unclear. However, ER− tumors are known to exhibit a more aggressive phenotype associated with lower rates of overall survival (Aamdal *et al.*, 1984) and would be expected to recur earlier and more frequently. Estrogenic effects of TAM in these women also could have increased the number of deaths from cardiovascular disease and stroke, reflecting the data noted above from recent studies of estrogenic HRT use (Viscoli *et al.*, 2001; WHI, 2002).

Antiestrogens and breast cancer chemoprevention

TAM's ability to inhibit contralateral breast cancers and relatively low incidence of serious side effects led to studies into its potential use as a chemopreventive agent for patients with a high breast cancer risk. Three large, randomized, chemoprevention studies with TAM have

been performed to date: the NSABP P-1 trial ($n=13\,388$ participants) (Fisher *et al.*, 1998), the Royal Marsden Trial ($n=2471$ participants) (Powles *et al.*, 1998), and the Italian Chemoprevention Trial ($n=5408$ participants) (Veronesi *et al.*, 1998). Outcomes have been mixed: no significant reduction in risk was seen in the initial reports of either the UK or Italian trials, whereas the P-1 trial reported significant reductions in the incidence of both noninvasive (50%) and invasive (49%) breast cancers. A recent update on the Italian Trial reports an 82% TAM-induced reduction in the breast cancer risk among women at high risk for ER+ breast cancer (Veronesi *et al.*, 2003). In the NSABP trial, reductions in breast tumor incidence were seen only in the incidences of ER+ tumors (Fisher *et al.*, 1998). Reasons for the disparities among the trials have been widely discussed; these tend to focus on differences in patient populations, subject eligibility criteria, and study size. Results from the NSABP P-1 trial, which are broadly consistent with the 39% reduction in contralateral breast cancer incidence reported for TAM use (EBCTCG, 1992), are usually considered the more definitive. These data contributed to the decision by the Federal Drug Administration (USA) in October 1998 to allow the use of TAM as a chemopreventive agent for breast cancer. More recently, NSABP has reported TAM-induced reductions in the risks of adenosis, fibrocystic disease, hyperplasia, metaplasia, fibroadenoma, and fibrosis in the P-1 trial (Tan-Chiu *et al.*, 2003).

Estrogens and breast cancer

Since antiestrogen action and resistance are intimately affected by estrogen exposure, we briefly address the role of estrogens in breast cancer. An association between parity and breast cancer risk was observed by the 16th century Italian physician Bernadino Ramazzini (1633–1714) in his '*De Morbis Artificium*' published in 1700. The ability of ovariectomy to induce remissions in premenopausal breast cancer patients was shown by the Scottish physician George Beatson, the first clear evidence of an effective endocrine therapy for this disease (Beatson, 1896). More recent epidemiologic data show clear associations of early age at menarche, late age at menopause (Nishizuka, 1992), pregnancy (Hsieh *et al.*, 1994), obesity (Hulka and Stark, 1995), serum estrogen concentrations (EHBCCG, 2002), and use of estrogenic HRTs (Magnusson *et al.*, 1999; Schairer *et al.*, 1999, 2000) or oral contraceptives (Berger *et al.*, 2000) with an increase in the risk of developing breast cancer. Risk appears related to the timing of exposure and whether the cancer develops during the premenopause or postmenopause (Hilakivi-Clarke *et al.*, 2002).

Precisely how estrogens affect breast cancer risk remains controversial and outcome may be dependent upon the timing and duration of exposure. During the postmenopausal years, estrogenic stimuli are more closely associated with an increased breast cancer risk. However, we have recently reviewed evidence consistent with the hypothesis that, depending on the timing of exposure, increased estrogenic exposure can be associated with a reduced risk of breast cancer (Hilakivi-Clarke *et al.*, 2002). For example, estrogenic stimuli during childhood or the premenopausal years may affect breast development such that the breast is less susceptible to transformation. Estrogens may reduce breast cancer incidence in some women by altering mammary gland development and inducing the expression of genes involved in DNA repair (Hilakivi-Clarke *et al.*, 1999a; Hilakivi-Clarke, 2000).

For the purposes of this review, we will focus on the aspects of estrogen exposure that are associated with increased breast cancer risk and the survival/proliferation of established neoplastic breast cells. Hence, estrogens can be considered to act either as promoters (factors that stimulate the growth and/or survival of existing transformed cells) or as initiators (factors that induce the genetic damage that leads to cellular transformation). Evidence that estrogens are tumor promoters is well established from both experimental and clinical observations. For example, the growth of several human breast cancer cell lines *in vitro* and *in vivo* is stimulated by estrogenic supplementation. Indeed, such estrogenic supplementation is effective whether administered as classical estrogens (e.g., estradiol, estrone, or estriol) or plant-derived phytoestrogens such as the isoflavone genistein (Hsieh *et al.*, 1998). In addition, antiestrogens, aromatase inhibitors, leutinizing hormone releasing hormone agonists/antagonists, and ovariectomy are effective in the treatment of some breast cancer patients, all of which limit the interaction between a promotional (estrogenic) stimulus and cancer cells.

As tumor promoters, the effects of estrogens are related to the duration and timing of exposure. Withdrawal of an estrogenic stimulus that acts as a promoter could produce an eventual reduction in risk because it no longer promotes the growth or survival of existing cancer cells. Pregnancy produces a natural and significant increase in circulating estrogens, but only a transitory increase in breast cancer risk in young women. Indeed, if the first pregnancy was at a young age, the short-term increase may eventually translate into a lifetime reduction in breast cancer risk (Hsieh *et al.*, 1994). The increased breast cancer risk associated with either oral contraceptive or estrogenic HRT use is also related to the recency of use. Risk begins to reduce with the cessation of use and is highest in current users (CGHFBC, 1996; Schairer *et al.*, 2000).

Evidence that estrogens act as chemical initiators is more controversial. Estrogens can exhibit carcinogenic activity in some animal models; perhaps the best-known example is the ability of estrogens to induce renal cancers in Syrian hamsters (Kirkman, 1972). However, compelling evidence that estrogens initiate mammary cancer in animals is hard to find. In the 1930s, Lacassagne (1932) performed several studies in male mice and showed that administration of large doses of estrone can induce mammary tumors. While consistent

with an estrogen-mediated initiation of mammary cancer, it is possible that the mice were infected with the mouse mammary tumor virus (MMTV). Other than some transgenic/null mouse models, only in the ACI rat does estrogen administration reproducibly produce a high incidence of mammary tumors (Cavalieri and Rogan, 2002).

Reactive estrogen semiquinone/quinone intermediates, produced by the redox cycling of estrogen metabolites hydroxylated at the C3 and C4 positions of the aromatic A-ring, are the most likely estrogen initiators (Cavalieri *et al.*, 1997; Bishop and Tipping, 1998; Cavalieri and Rogan, 2002). These reactive species can generate a substantial intracellular oxidative stress and directly damage DNA through the production of DNA adducts. Such events could define reactive estrogen metabolites as initiators, rather than as merely promoters of carcinogenesis. Recently, the National Toxicology Program (2003) listed, for the first time, steroidal estrogens as carcinogens.

Estrogen independence and antiestrogen resistance

Estrogen independence and antiestrogen resistance are often considered to be synonymous, which is not surprising since ER− tumors are definitively estrogen-independent and very rarely respond to antiestrogens, ovariectomy, or aromatase inhibitors. Nonetheless, several observations suggest that various forms of both estrogen independence and antiestrogen resistance exist and that these may be biologically and clinically very different. For example, second-line responses to aromatase inhibitors after response and recurrence on TAM are common (Goss *et al.*, 1995; Buzdar *et al.*, 1996). Crossover between more similar compounds, such as other nonsteroidal antiestrogens, rarely produces secondary responses (Johnston, 2001), although crossover to structurally different antiestrogens can produce secondary responses in patients. Tumors that respond first to TAM (triphenylethylene) show a marked response to ICI 182,780 (steroidal) administered upon failure of the TAM therapy (Howell *et al.*, 1995). Similar patterns of responses were seen previously in experimental models (Brünner *et al.*, 1993b). For example, MCF-7 human breast cancer cells were selected for the ability to grow in the absence of estrogens (Clarke *et al.*, 1989a). The selected cells are estrogen-independent because they no longer require estrogens for growth either in cell culture or as xenografts in athymic nude mice. However, when exposed to either 4-hydroxytamoxifen or ICI 182,780, the cells are growth inhibited both *in vitro* and *in vivo* (Clarke *et al.*, 1989a; Brünner *et al.*, 1993a, b).

These observations strongly imply that the ability of breast cancer cells to grow in a low or nonestrogenic environment is not always synonymous with antiestrogen resistance. Four antiestrogen resistance phenotypes have been defined (Clarke and Brünner, 1995) and are shown in Table 1. The clinical applicability of these phenotypes remains to be determined but they are useful for defining resistance phenotypes in experimental models.

Intratumor estrogens and antiestrogens and exogenous estrogenic exposures

Antiestrogens act within cells, primarily to compete with available estrogens for binding to ER. Thus, the antiestrogenic potency of any compound is related to its affinity for ER relative to that of any estrogens present and the concentrations of both the antiestrogens and estrogens. The data in Table 2 show the relative affinities of the primary estrogens, antiestrogens and their major metabolites, and selected environmental estrogens and phytoestrogens. Intratumor estrogen concentrations are affected by several factors including serum estrogen concentrations and local estrogen production within the breast. Serum estrogen concentrations are affected by the presence or absence of functional ovaries and exogenous estrogen use such as HRT, some oral contraceptives, and various dietary components.

Passive diffusion into cells across the plasma membrane appears to be TAM's and estradiols's primary method of entry into cells. However, both TAM and estrogens are extensively bound to serum proteins and probably also to cellular proteins in tumor/nontumor cells within the breast (Clarke *et al.*, 2001b). Release from serum proteins likely occurs within the tumor vasculature, with both estrogens and antiestrogens being subsequently sequestered within tumor/nontumor cells by intracellular proteins. The lipophilicity of both hormone and drug, and the significant amount of adipose tissue in the breast, may produce a local reservoir for both estrogens and antiestrogens. However, the concentration of free drug/hormone within cells and serum may be relatively low. Intracellular sequestration of drug/hormone in tumor and stromal cells could produce a concentration gradient favoring

Table 1 Antiestrogen resistance phenotypes

Antiestrogen resistance	*Phenotype*
Type 1	Fully responsive to antiestrogens and aromatase inhibitors
Type 2	Resistant[a] to nonsteroidal antiestrogens but responsive to ICI 182,780 and aromatase inhibitors (or resistant to ICI 182,780 but responsive to nonsteroidal antiestrogens and aromatase inhibitors)
Type 3	Resistant to all antiestrogens but potentially responsive to aromatase inhibitors
Type 4	Multihormone-resistant (resistant to all endocrine therapies and includes ER− and PR− tumors)

[a]Resistance can be considered as unresponsiveness and antiestrogen-stimulated phenotypes

Table 2 Relative binding affinities (approximate) of selected estrogens, antiestrogens, and environmental estrogens and phytoestrogens[a]

Compound	*Relative binding affinity (17β-estradiol = 100)*	
	ERα	*ERβ*
Estrogens		
Estrone	60	37
Estriol	14	21
Antiestrogens		
Tamoxifen	7	6
4-Hydroxytamoxifen	178	339
Nafoxidine	44	16
ICI 164,384[b]	85	166
Raloxifene	69	16
Clomiphene	25	12
Environmental estrogens and phytoestrogens		
Genistein	5	36
Resveratrol	$<1.1 \times 10^{-4}$	$<1.6 \times 10^{-4}$
Zearalenol	7	5
o,p'-DDE 2(2-chloro-phenyl)-2-(4-chlorophenyl)-1,1dichloroethylene	<0.01	<0.01
Bisphenol A	0.01	0.01

[a]Adapted from Kuiper *et al.* (1998), Kuiper *et al.* (1997) and Bowers *et al.* (2000); the methods for estimating ER binding are not the same across these studies but all three express binding relative to the values estimated for 17β-estradiol. [b]ICI 182,780 is an analog of ICI 164,384

diffusion into local tissues. If the affinity and capacity of tissue for drug/hormone exceed that of blood, significant accumulation within tumors would likely occur. Data in Table 3 (adapted from Clarke *et al.*, 2001b) illustrate several points regarding the pharmacokinetics of estrogens and antiestrogens. For example, intratumor concentrations of both estradiol and TAM are much higher than their respective concentrations in the serum. For estrogens, where the primary estrogen present in tumors is 17β-estradiol, both biosynthesis within the tumor and significant uptake from blood occur.

The ability of estrogens and antiestrogens to compete for binding to ER is likely to reflect intracellular availability. While their respective free concentrations are largely unknown, the data in Tables 2 and 3 imply that many breast tumors should accumulate a sufficient excess of TAM and its major antiestrogenic metabolites to compete readily with intratumor estrogens. If the estimate for estradiol concentrations (1.29 nM) and the reported concentrations for TAM and its major metabolites ($\sim 3\,\mu$M TAM + $\sim 7\,\mu$M *N*-desmethyltamoxifen + $\sim 0.2\,\mu$M 4-hydroxytamoxifen) in tumors are good approximations (Table 3), antiestrogenic metabolites may accumulate to levels up to 10^4-fold higher than estradiol. While TAM and *N*-desmethyltamoxifen have relative ER binding affinities about 10% that of estradiol (Table 2), overall, antiestrogenicity may exceed estrogenicity in most TAM-treated breast tumors by 100-fold (assuming equivalent availability).

This interpretation is consistent with the initial antiestrogenic activity of TAM seen in most ER+ breast cancers. No compelling evidence shows that TAM becomes extensively metabolized to purely estrogenic metabolites in patients with antiestrogen-resistant cancer. Furthermore, little evidence has been produced to suggest that the balance of TAM metabolism is such

Table 3 Serum and intratumor estrogen and tamoxifen concentrations[a]

Serum concentrations		
Mean estimates of estrogen concentrations		*Comments*
Follicular phase	Luteal phase	
≤0.28 nM	≤1.1 nM	Normal menstrual cycle
Pregnancy		
≤150 nM		Normal third trimester (when estrogen concentrations are highest)
Breast cancer	Controls	
0.114 nM	0.093 nM	All postmenopausal women; in most studies these differences are statistically significant[1]
Estimates of tamoxifen concentrations		
Concentration	Drug/metabolite	
≤1.1 μM	Tamoxifen + metabolites	Similar to normal tamoxifen regimen
≤4.0 μM	Tamoxifen	High-dose tamoxifen regimen
≤6.0 μM	*N*-desmethyltamoxifen	High-dose tamoxifen regimen
Intratumor concentrations		
Mean estimates of estrogen concentrations		*Comments*
Breast tumors	Non-neoplastic	
1.29 nM	0.76 nM	Non-neoplastic includes adjacent normal, fibroadenomas, adipose tissues
Mean estimates of tamoxifen concentrations		
Concentration	Drug/metabolite	Mean estimates vary across studies. The values represented here are among the higher of the reported mean values[a]
≤3.0 μM	Tamoxifen	Breast tumors
≤4.0 μM	Tamoxifen	Brain metastases from breast cancer
≤7.0 μM	*N*-desmethyltamoxifen	Breast tumors
≤8.0 μM	*N*-desmethyltamoxifen	Brain metastases from breast cancer
≤0.2 μM	4-Hydroxytamoxifen	Brain metastases from breast cancer

[a]See Clarke *et al.* (2001) as to how these values were obtained and for citations to the source publications

that sufficient concentrations of its estrogenic metabolites are produced to overcome TAM's intracellular cumulative antiestrogenicity (combination of parent drug plus its antiestrogenic metabolites) (Clarke *et al.*, 2001b). Currently, no clinically relevant ER variants/mutants have been described that could adequately affect intratumor pharmacology to an extent sufficient to offset this balance in favor of a TAM-stimulated or other antiestrogen-resistant phenotype in a significant proportion of breast cancers.

Changes in TAM influx/efflux could alter its intracellular concentrations, and limited evidence suggests that this may occur in some tumors. However, the extent to which it occurs and the mechanisms driving such changes are unclear (Clarke *et al.*, 2001b).

Exogenous estrogenic exposures and their effects on antiestrogen resistance

Since estrogens compete with antiestrogens for ER binding, any compound with either estrogenic activity or the ability to increase estrogen exposure could affect response to antiestrogens. Estrogenic exposures come in many forms, including plant and environmental estrogens (Hilakivi-Clarke *et al.*, 1999b; Clarke *et al.*, 2001a), dietary exposures that affect the levels of endogenous estrogens (Hilakivi-Clarke *et al.*, 1997), and estrogenic HRT (Clarke *et al.*, 2001b). Dietary antioxidant exposure also may affect antiestrogen responsiveness (Clarke *et al.*, 2001b) and some women already take the most potent natural antioxidant (vitamin E) as an alternative medicine for controlling menopausal symptoms (Stampfer *et al.*, 1993; Barton *et al.*, 1998; Koh *et al.*, 1999).

The inclusion of women on HRT in some of the chemoprevention trials has been one of the issues raised to explain the lack of TAM's activity in these trials. It is unlikely that HRT would raise serum estrogens beyond levels seen in TAM responsive premenopausal women. However, the nature of the estrogenic exposure is very different between postmenopausal women on HRT and premenopausal women. More data are required to assess directly the contribution of HRT to TAM responsiveness.

Dietary exposures and tamoxifen activity

Several dietary components, including those present in dietary fats, soy, fruits, vegetables, and alcohol, have been suggested to have either protective or harmful effects on the breast. Some of these dietary factors, such as dietary fats and soy, can alter circulating estrogen levels (Lu *et al.*, 2000) and interact with ER (Wang *et al.*, 1996b; Collins *et al.*, 1997; Zava and Duwe, 1997). TAM's ability to affect the growth of ER+ tumor cells may be altered by dietary intakes of fats and soy. Fats, soy, and other dietary components also modify other cell signaling pathways (Agarwal, 2000; Bouker and Hilakivi-Clarke, 2000; Clarke *et al.*, 2002). If TAM signals through the same pathways, a dietary factor might modify TAM's ability to inhibit the growth of malignant breast cells (ER-dependent or -independent interactions). Dietary components that alter signaling of a pathway that affects tumor growth independent of TAM also could either potentiate or reverse TAM's effects. Data from both *in vitro* and *in vivo* studies strongly support the hypothesis that at least some dietary factors modify TAM's actions in the breast.

Soy, dietary fat, vegetables, and antiestrogen responsiveness

High soy protein intake has been proposed to contribute to low breast cancer incidence among Asian women (Adlercreutz, 1995). A recent meta-analysis shows that a high intake of soy is associated with a reduced risk of developing premenopausal, but not postmenopausal, breast cancer (Trock *et al.*, 2001). Soybeans contain large amounts of the isoflavones daidzein and genistein (Barnes *et al.*, 1994; Adlercreutz, 1995). Genistein has many biological effects that could potentially reduce breast cancer risk, including inhibition of tyrosine kinase, EGFR tyrosine phosphorylation, and topoisomerase II activities. It also arrests cell cycle progression at G_2–M, induces apoptosis, has antioxidant properties, modifies eicosanoid metabolism, and inhibits *in vitro* angiogenesis (see the review by Messina *et al.*, 1994). While each of these actions of genistein could influence antiestrogen responsiveness, they occur primarily at pharmacologic rather than physiologic exposures. Humans consuming high levels of soy-based food products have less than 1 μM of circulating genistein (Messina *et al.*, 1994), and 30–185 μM genistein is required to induce many of the above-mentioned effects in experimental models *in vitro* where bioavailability is already likely to be greater than *in vivo*.

At physiological concentrations, genistein exhibits estrogenic properties that could enhance breast cancer risk. Genistein activates the ER (Wang *et al.*, 1996b; Collins *et al.*, 1997; Zava and Duwe, 1997) and induces proliferation of human breast cancer cells *in vitro* (Martin *et al.*, 1978; Wang *et al.*, 1996b). Genistein also stimulates proliferation of mammary epithelial cells in rodents (Santell *et al.*, 1997; Hsieh *et al.*, 1998) and in women (Petrakis *et al.*, 1996; McMichael-Phillips *et al.*, 1998). Data from ovariectomized athymic mice, representing a model of postmenopausal breast cancer, show that genistein and soy protein isolate both promote the growth of MCF-7 xenografts (Allred *et al.*, 2001). Furthermore, a recent study in athymic mice showed that genistein blocked the inhibitory effect of TAM on the growth of MCF-7 xenograft (Ju *et al.*, 2002). These results suggest caution in consuming high levels of genistein among postmenopausal women who are taking TAM for their breast cancer or to reduce their risk of developing breast cancer.

Very little is known about possible interactions between high dietary fat intake and the activity of TAM. TAM has beneficial effects on some aspects of fatty acid metabolism, for example, by reducing cholesterol levels (Reckless *et al.*, 1997). Diets containing n-3 PUFAs can increase the efficacy of cytotoxic

drugs against ER− human breast cancer xenografts (MDA-MB-231) (Hardman *et al.*, 2001). A recent study suggests that n-3 PUFAs restore TAM's ability to inhibit cell growth (DeGraffenried *et al.*, 2003). Oleic acid appears to affect indirectly TAM's dissociation from cellular antiestrogen binding sites (Hwang, 1987), an effect that could increase the intracellular concentrations of free drug. Since n-3 PUFAs have many biological activities, they may play a role in modifying TAM's actions, including an ability to inhibit protein kinases (Mirnikjoo *et al.*, 2001). γ-linolenic acid has several properties that might make it antitumorigenic. Kenny *et al.* (2001) have shown that γ-linolenic acid reduces the growth of MCF-7 xenografts, reduces ER levels in these cells, and potentiates TAM's ability to inhibit cell growth. However, the precise mechanism of action of γ-linolenic acid remains to be determined.

Cruciferous vegetables, such as broccoli, cabbage, cauliflower, and brussel sprouts contain high levels of indole-3-carbinol (I3C) and its metabolite 3,3-diindolymethane (DIM). These compounds have been shown to exhibit chemopreventive activity in multiple target organs including the breast (Bradlow *et al.*, 1999). Several mechanisms of action have been proposed for I3C and DIM, including changes in phase I and II enzyme activities and in cell cycle progression. Data from Katchamart and Williams (2001) show that I3C and DIM downregulate the expression of the cytochrome *P*-450 components that convert TAM to its more potent metabolites. Thus, these authors propose that high intake of cruciferous vegetables might reduce TAM efficacy. Vitamin A/retinoids can interact with estrogens, and some studies suggest that retinoids can increase the activity of TAM (McCormick and Moon, 1986; Anzano *et al.*, 1994). Little evidence from human studies exists to support directly this interaction. However, remarkably few studies have been undertaken in this area and additional data are clearly needed.

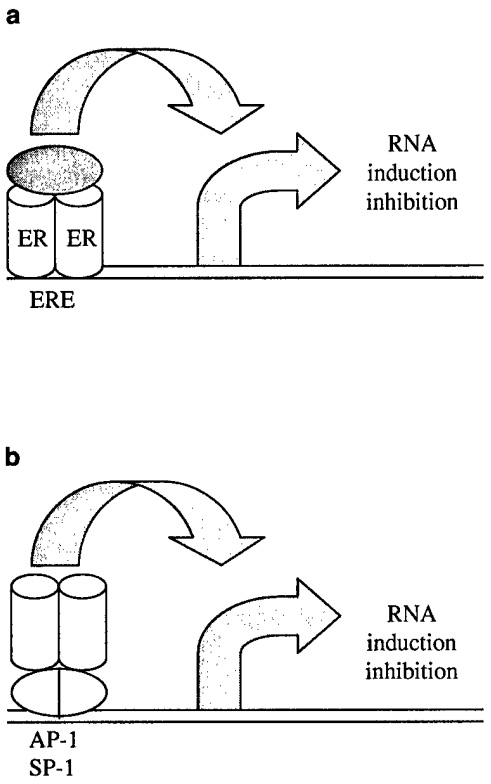

Figure 1 Estrogen receptor (ER) function—a simplistic representation. ERs function as nuclear transcription factors, bound to either estrogen responsive elements (**a**) or to proteins bound to other responsive elements, for example, AP-1, SP-1 (**b**). Transcription can be induced or repressed, with the pattern of genes affected likely reflecting the mix of coregulators available to bind to the various ER-transcription complexes formed on respective promoters. Evidence for both ligand-dependent and -independent activation exists, and it is clear that different ligands can induce different conformations in the bound ER proteins. ER = estrogen receptor; in (**a**) the hatched elipse represents a coregulator; in (**b**) the split elipse represents a protein complex such as AP-1 or SP-1

Estrogen receptors and antiestrogen resistance

Two ER genes have been identified: the classical ERα on human chromosome 6q25.1 and ERβ on chromosome 14q22–25. Each receptor acts as a nuclear transcription factor that binds responsive elements (estrogen responsive elements; EREs) within the promoters of target genes (Figure 1a) or binds to other proteins and affects their abilities to regulate transcription (e.g., AP-1, SP-1; Figure 1b). ERα and ERβ homology is limited in the transcriptional regulatory domains, particularly in the N-terminal region. Both ER homodimers and heterodimers are formed and these may differ in their ability to affect transcription at some promoters (Tyulmenkov *et al.*, 2000). For example, the ER binds directly to EREs, which are broadly defined consensus sequences with some tolerance to variation in their sequence. ER also binds to, and regulates the transcriptional activation of, other transcription factors including AP-1, SP-1, and at cyclic AMP response elements (CRE) (Paech *et al.*, 1997; Castro-Rivera *et al.*, 2001; Liu *et al.*, 2002b).

The patterns of ER expression vary in the mammary gland. In most normal mammary epithelia, the two receptors are rarely expressed in either a high proportion of cells or at very high levels. The ERα:ERβ ratio may change during carcinogenesis, such that the ERα proportion increases as the cells acquire a more progressed phenotype. Whether this change reflects an increase in ERα or a decrease in ERβ expression (Leygue *et al.*, 1998), and whether it is a function or a consequence of malignant transformation or progression is unclear. ERα appears to be the more highly expressed of the two receptors in breast tumors (Leygue *et al.*, 1998; Speirs *et al.*, 1999a), at least when both are coexpressed in the same cells (Saunders *et al.*, 2002). However, some of the few existing studies that measured both ERα and ERβ proteins have been complicated by the use of different antibodies of occasionally uncertain quality (Speirs, 2002).

When occupied by estradiol, ERα and ERβ can produce similar effects on gene regulation in simple

ERE-driven reporter construct studies (Kuiper *et al.*, 1996). However, the ligand binding profiles of the two receptors may be species specific (Harris *et al.*, 2002). Furthermore, at other promoters, the two receptors have very different activities. For example, ERα and ERβ have opposite effects on transcription driven by AP-1, SP-1, or CRE sites in promoter–reporter assays (Paech *et al.*, 1997; Castro-Rivera *et al.*, 2001; Maruyama *et al.*, 2001a; Liu *et al.*, 2002b). Differential regulation of cyclin D1 by ERα and ERβ has been reported (Liu *et al.*, 2002b), and ERβ can block the transcriptional activation of AP-1 by ERα (Maruyama *et al.*, 2001b). Changes in ER expression/activation might be important in affecting endocrine responsiveness if genes driven primarily by AP-1, SP-1, and/or CRE elements are rate limiting in affecting signaling to apoptosis/proliferation/survival.

The relative importance of ERα and ERβ in affecting antiestrogen responsiveness remains to be established. However, the extensive existing data with well characterized ERα antibodies that do not recognize ERβ allow for some speculation. Ligand binding ER assays (do not differentiate between ERα and ERβ) and immunohistochemical detection of ER in patients' tumors (detect ERα only) broadly agree in their determination of ER-positivity and prediction of TAM sensitivity (Alberts *et al.*, 1996; Molino *et al.*, 1997). Thus, whatever the role of ERβ, measuring ERα is sufficient to predict whether or not a patient is likely to benefit from treatment with antiestrogen, aromatase inhibitor, or ovariectomy. These findings also would be consistent with a requirement of ERα for antiestrogen sensitivity, which is further consistent with data from most experimental models in which ERα is usually the dominant ER isoform expressed.

Since loss of ERα (i.e., the tumor phenotype changes from ERα+ to ERα−) is relatively uncommon as an acquired antiestrogen resistance mechanism, it seems unlikely that many resistant tumors acquire a true ERα−/ERβ+ phenotype. If there is a role for ERβ, it may be driven by changes in its expression level relative to ERα, since heterodimers are functionally important (Pettersson *et al.*, 1997; Tyulmenkov *et al.*, 2000). When introduced into ER− MDA-MB-231 breast cancer cells, ERβ produces ligand-independent inhibition of proliferation, whereas ERα-mediated effects are ligand-dependent (Lazennec *et al.*, 2001). A ligand-independent suppression of growth by ERβ might confer a multihormone-resistant phenotype (Schinkel *et al.*, 1991) (multihormone resistance is Type 4 resistance as shown in Table 1), since ICI 164,384 could not block the ligand-independent effect of ER expression in MDA-MB-231 cells (Lazennec *et al.*, 2001).

Currently, determining the relative importance of ERβ expression in antiestrogen responsiveness is limited by the lack of adequate data regarding ERβ protein expression in responsive and resistant breast tumors. The possible association of ERβ mRNA expression with a poor prognosis (Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999b) may further complicate matters. Only one small study ($n=9$ TAM resistant; $n=8$ TAM responsive tumors) has explored the association of ERβ expression with antiestrogen resistance. The authors reported increased ERβ mRNA expression in antiestrogen-resistant tumors (Speirs *et al.*, 1999a). Nonetheless, the outcome is potentially confounded by the very small number of cases, the fact that only ERβ mRNA was measured, and the possible association of ERβ expression with a more aggressive phenotype (Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999b).

Several mutant and splice variant forms of both ERα and ERβ have been reported and previously reviewed (Hopp and Fuqua, 1998; Murphy *et al.*, 1998). Compelling evidence that any of these are functionally relevant in driving a significant proportion of breast cancers remains largely unconvincing. For example, most data only measure mutant mRNAs that may not be translated into biologically relevant protein concentrations in cells. Most tumors that express mutant ER concurrently express the wild-type receptor, with the mutant representing a relatively small proportion of total ER. A mutant ERα (D351Y) that perceives TAM as an agonist has been described in some TAM-stimulated MCF-7 cell variants (Jiang *et al.*, 1992). Similarly, changes in the F-region of the receptor also can affect the activities of estradiol and 4-hydroxytamoxifen (Schwartz *et al.*, 2002). The agonist activities of raloxifene are also increased in D351Y (Liu *et al.*, 2002a). Expression of this mutant in breast tumors in patients has not been reported. Thus, the clinical relevance of this ER mutant or functionally similar ER mutant proteins remains unclear. However, our understanding of the role of ER mutants and variants may change in the near future (Fuqua, 2001). Currently, little compelling evidence exists in support of mutant or splice variant ERα and/or ERβ contributions to either *de novo* or acquired antiestrogen resistance or hormone independence (Karnik *et al.*, 1994; LeClercq, 2002). However, the importance of receptor mutations and varinats in other diseases suggests that a role for these modifications of ERs may yet be shown to be important.

Coregulators of estrogen receptor function and antiestrogen resistance

Whatever the ERE and/or other transcription factor bound, the ability to affect transcription of a target gene is further modified by multiple components of the transcription complex. Perhaps the most widely studied modifiers of ER-mediated transcription are the coregulators. Coregulators can be either coactivators (inducers) or corepressors (inhibitors) of gene transcription. These molecules often act by altering histone acetylation (Kim *et al.*, 2001). While most studies of coregulator action have been carried out with ERα, ERβ function is also affected (Tremblay *et al.*, 1998), as is the activity of other members of the steroid hormone receptor superfamily.

ER coregulators in several protein families have been described in recent years, almost all of which are ubiquitously expressed (Graham *et al.*, 2000) and defined initially by their ability to affect ER-mediated

transcription in simple promoter–reporter transcription assays. Considerable redundancy is evident, with many coactivators or corepressors exhibiting similar transcription regulatory effects in comparable/identical biological assays. A full understanding of the role of coregulators may be further complicated by gene promoter-, tissue-, and species-specific effects, all of which contribute to the cellular context. Thus, the pattern of other proteins expressed in a cell (cellular context) may greatly influence how and whether a specific coregulator is the dominant effector in regulating a ligand's ability to affect ER-mediated transcription (Clarke and Brünner, 1996; Clarke *et al*., 2001b).

The ability of an ER-driven transcription complex to recruit coregulators can be strongly ligand-dependent. For example, 4-hydroxytamoxifen induces a conformation that blocks the coactivator recognition groove in ER (Shiau *et al*., 1999). Estrogens and antiestrogens have long been known to affect the physical properties of ERs (Miller *et al*., 1984). The importance of ligand to receptor conformation and activation led to early conceptual models that have received renewed attention in recent years. Perhaps the most important information has come from crystallographic studies of the ER binding domain complexed with different ligands (Brzozowski *et al*., 1997; Pike *et al*., 1999; Shiau *et al*., 2002). Several laboratories have used these data to describe conceptually similar models of ER function when liganded with either agonists or antagonists (Wurtz *et al*., 1998; Pike *et al*., 1999; Liu *et al*., 2002a, Shiau *et al*., 2002). The major limitations of such studies are the use of only the ligand binding domain (requires the assumption that no other domains of the ER affect its structure) and the use of crystal structures that may or may not fully reflect receptor structure in the more complex environment of a living cell. Nonetheless, data from such studies can provide important molecular insights into important biological responses.

The consequences of ligand-specific ER conformations are becoming evident but may be complex (McKenna *et al*., 1999). The coactivator SRC-1 produces a ligand-independent activation of ER while enhancing the agonist activity of the potent TAM metabolite 4-hydroxytamoxifen (Smith *et al*., 1997). SRC-1 also interacts synergistically with CRE binding proteins in regulating ER-mediated transcription (Smith *et al*., 1996). SMRT (corepressor) binds ER and blocks the agonist activity of 4-hydroxytamoxifen induced by SRC-1 (Smith *et al*., 1997). N-CoR is a corepressor that binds TAM-occupied but not ICI 182,780-occupied ER (Jackson *et al*., 1997). The functional relevance of this latter observation is consistent with the lack of full crossresistance between these two drugs in cell cultures models (Brünner *et al*., 1993b) and in breast cancer patients (Howell *et al*., 1995; Robertson, 2001). However, a recent study found no association between N-CoR expression and outcome in TAM-treated patients (Osborne *et al*., 2002).

It might be expected that increased expression or function of a protein that allows an antiestrogen to act as an agonist, or decreased expression of a coregulator that suppresses ER activity when the receptor is occupied by an antiestrogen, could confer a degree of antiestrogen resistance (Clarke and Brünner, 1996; Clarke *et al*., 2001b). Evidence for this in human cancers and experimental models remains somewhat limited. Expression of the corepressor N-CoR is lower in TAM-stimulated MCF-7 xenografts than in wildtype xenografts (Lavinsky *et al*., 1998), but the functional relevance of the observation in human cancers is unclear. Chan *et al*. (1999) studied a small cohort of TAM-resistant human breast tumors ($n=19$) but found no difference in the expression of TIF-1, RIP140, or the corepressor SMRT. Lower levels of the coactivator SUG-1 were detected in some TAM-resistant tumors, but the consequences for antiestrogen responsiveness of reduced SUG-1 expression require further study.

Extrapolating many of these observations to specific biological functions in breast tumors is not always a simple matter. For example, most data have been obtained, of necessity, from the use of somewhat artificial experimental models with simple promoter conformations. ERE structure is variable across known estrogen-regulated genes, and a promoter's ability to bind ERs and coregulators can be affected by its local structure (Truss and Beato, 1993; Nardulli *et al*., 1995; Lee and Lee, 2001). Different ER–antiestrogen complexes also may recognize different promoter elements (Yang *et al*., 1996). Thus, promoter context is likely to be important (Clarke and Brünner, 1996). Given the evidence of considerable coregulator redundancy and ubiquitous expression (McKenna *et al*., 1999; Planas-Silva *et al*., 2001; McKenna and O'Malley, 2002), it is unclear whether measuring or affecting changes in the expression/function of any single coregulator will prove clinically useful. Attempting to affect resistance by modifying the expression of any single coregulator could be confounded by compensatory responses in other coregulators, as likely happens for mammary gland development in SRC-1 (Xu *et al*., 1998) and E6-AP null mice (Smith *et al*., 2002). A greater degree of specificity will likely be obtained by targeting specific genes within a functionally relevant gene network (Clarke and Brünner, 1996), which would be downstream of any coregulator activities. The overall balance in the patterns and levels of expression of coactivators and coregulators also likely contributes to ER signaling and endocrine responsiveness. Clearly, cellular context is critical in assessing the role of specific coregulators in affecting a given phenotype (Clarke and Brünner, 1996; Clarke *et al*., 2001b).

In summary, with such redundancy and apparent lack of cell/tissue specificity, measuring the expression of specific coregulators to predict an antiestrogen-resistant phenotype may be uninformative, and affecting changes in the expression/function of any single coregulator to alter phenotype may prove difficult. We still do not know with any certainty which estrogen-regulated genes are responsible for affecting cell proliferation, cell survival, or apoptosis in breast cancer. Hence, we do not know the structure of their promoters, the coregulators their occupied receptors can recruit into

functional or inactive transcription complexes, or the cellular context in which they exist in responsive and resistant cells.

Estogen receptor-independent cell signaling in antiestrogen resistance

Only a small proportion of ER−/PR− tumors respond to antiestrogens, consistent with their primary actions being mediated by ER. Nonetheless, many investigators have explored ER-independent signaling as mechanisms of antiestrogen resistance. The primary role of these effects is unclear and some occur at concentrations that are not pharmacologically relevant. Nonetheless, such activities can alter ER function or may interact with signaling downstream of ER (Figure 2). Since these mechanisms have been reviewed in detail (Clarke *et al.*, 2001b), we now only briefly discuss some of the more relevant.

Antiestrogen-induced induction of oxidative stress responses is perhaps the most widely studied ER-independent mechanism. The redox metabolism of several TAM metabolites can give rise to reactive species that can induce oxidative stress (Ye and Bodell, 1996), and both TAM and 4-hydroxytamoxifen produce 8-hydroxy-2′deoxyguanosine (Okubo *et al.*, 1998). TAM's ability to induce quinone reductase (Montano and Katzenellenbogen, 1997), protein kinase C redistribution (Gundimeda *et al.*, 1996), and lipid peroxidation (Schiff *et al.*, 2000), and our observations that antiestrogen-resistant cells upregulate cytochrome *c* oxidases (Gu *et al.*, 1997) and NFκB (Gu *et al.*, 2002) also are consistent with antiestrogen effects on oxidative stress responses (reviewed by Clarke *et al.*, 2001b).

Other ER-independent effects include perturbations in membrane structure (Clarke *et al.*, 1990b), changes in protein kinase C activation and subcellular localization (O'Brian *et al.*, 1986; Gundimeda *et al.*, 1996), and inhibition of the intracellular Ca^{++} binding protein calmodulin (Rowlands *et al.*, 1995). Some of these effects may be inter-related, since inhibition of protein kinase C also blocks calmodulin-dependent EGFR transactivation (Tebar *et al.*, 2002). These latter mechanisms may arise independent of ER, but would affect ER-mediated signaling. Calmodulin has been implicated as a coregulator of ER action (Biswas *et al.*, 1998), and EGFR-mediated signaling through MAPK may affect ER activation (see for recent reviews Clarke *et al.*, 2001b; Santen *et al.*, 2002).

The extent to which these mechanisms are truly ER-independent, in that they do not affect any aspect of ER-mediated signaling, requires further study. As with TAM's effects on calmodulin, ER-independent interactions may have significant effects on ER activation and function. For example, several growth factors appear to be able to activate ER through the induction of MAPK activities capable of changing ER's phosphorylation status (Clarke *et al.*, 2001b; Santen *et al.*, 2002). Other ER-independent events may interact with ER-mediated signaling downstream of ER activation. Despite these many activities, ER expression is required for most cells to respond to antiestrogens. While the importance of ER-independent signaling is unclear, many such signals may be necessary but not sufficient for affecting antiestrogen responsiveness (Clarke *et al.*, 2001b).

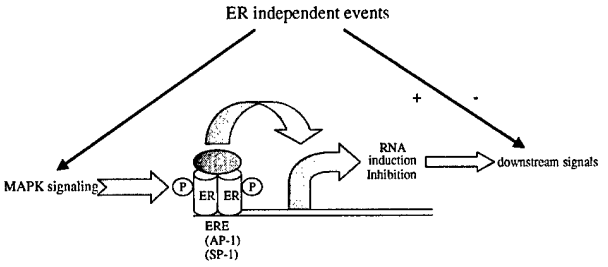

Figure 2 Putative role of estrogen receptor-independent effects of steroids and antiestrogens. These activities are induced by hormones or antihormones that are not directly mediated by their interactions with ERs. Such effects may be necessary, but they are not generally sufficient, to elicit a proliferative/antiproliferative response at most physiologically or pharmacologically relevant concentrations. ER-independent events may affect ER signaling either by altering ER activation and/or regulating the expression/function of other genes/proteins that are induced/repressed downstream of directly ER-regulated transcriptional events. The hatched elipse represents a coregulator; Ⓟ = phosphorylation

Antiestrogens, apoptosis, and cell death

Antiestrogenic exposures produce a G_0/G_1 cell cycle arrest (Taylor *et al.*, 1983), whereas estrogenic exposures are primarily mitogenic and increase the proportion of cells in S and G_2/M while reducing the proportion in G_0/G_1. Such effects are generally consistent with a cytostatic rather than cytotoxic effect. However, in our experience, long-term selection against antiestrogens *in vitro* or prolonged estrogen withdrawal from estrogen-dependent cells also induces cell death. Similar effects are seen in animal models. These observations are consistent with the ability of antiestrogens to reduce the incidence of ER+ breast cancers in high-risk women (chemoprevention) and produce an overall survival benefit in breast cancer patients (treatment). Initially, antiestrogens may produce a cytostatic effect that, in the longer term, results in cell death.

The precise mechanisms signaling to and responsible for antiestrogen-induced cell death are not fully understood. Most studies are consistent with an induction of an apoptotic or programmed cell death (Kyprianou *et al.*, 1991; Huovinen *et al.*, 1993; Zhang *et al.*, 1999). However, many breast cancers that acquire antiestrogen resistance still respond well to cytotoxic drugs, many of which also signal to apoptosis (Wang *et al.*, 1996a). Such effects could not occur if the machinery for inducing apoptosis was no longer intact or functional. Thus, the effects of antiestrogens must be upstream of effector mechanisms and reflect subtle changes in how ERs affect signaling to apoptosis. Other signaling pathways also may be important. Data from a recent study suggest

that adjacent normal mammary cells can induce cell death through Fas signaling in breast cancer cells. Resistance to this effect in some breast cancer cells was restored by inhibition of NFκB and PI3 kinase (Toillon *et al.*, 2002).

Tamoxifen-stimulated phenotype in antiestrogen resistance

While antiestrogens can induce growth arrest and apoptosis, in some patients, initiation of TAM therapy is associated with rapid progression of their disease, although continuation of TAM generally produces a beneficial response (Plotkin *et al.*, 1978; Clarysse, 1985). This response is called 'tumor flare' and is generally attributed to the estrogenic properties often seen with low doses of TAM. TAM takes approximately 4 weeks to reach effective steady-state levels, producing a window in which patients are exposed to suboptimal and potentially estrogenic concentrations of TAM (Buckely and Goa, 1989; Etienne *et al.*, 1989). These tumors are clearly not resistant to TAM, in either the pharmacologic or clinical context. Tumor flare should not be confused with the clinical TAM-stimulated resistance phenotype that may occur after prolonged TAM exposure and an initial TAM response.

Unlike tumor flare in previously untreated patients, evidence from MCF-7 human breast cancer xenografts suggests that some breast cancers may be initially growth inhibited by TAM, only to later become dependent on TAM for proliferation (Osborne *et al.*, 1987; Gottardis *et al.*, 1989; Connor *et al.*, 2001). These xenografts also retain the ability to be stimulated by estrogens (remain estrogen-dependent). Pharmacologically, this phenotype is not a resistance phenotype because the cells are clearly responding to the drug. However, a TAM-stimulated phenotype would represent clinical drug resistance because the nature of the response has changed in a manner that supports disease progression and would require a change in treatment. Acquired TAM dependence appears to reflect a switch in how the cells perceive TAM (as an ER agonist rather than antagonist). Several possible mechanisms may explain how this switch occurs in MCF-7 cells, including immunologic effects, ER mutations, and changes in growth factor or coregulator expression.

AIB1 and tamoxifen-stimulated growth as an antiestrogen resistance mechanism

AIB-1 (amplified in breast cancer-1; also known as SRC-3, RAC3, TRAM-1, pCIP, ACTR) is a steroid hormone receptor coactivator located on chromosome 20q12 (Anzick *et al.*, 1997) that has recently received attention as a possible contributor to antiestrogen responsiveness. AIB1 binds ER (Azorsa *et al.*, 2001), enhances the expression of cyclin D1 (Planas-Silva *et al.*, 2001), and exhibits somatic instability in some breast cancers (Dai *et al.*, 2002). AIB1's function as an ER coactivator produces increased transcriptional activation of ER (Anzick *et al.*, 1997). A novel AIB1 isoform (AIB-Δ3) has been recently reported that increases hormone and growth factor sensitivity (Reiter *et al.*, 2001) and increases the estrogenicity of 4-hydroxytamoxifen to a greater degree than wild-type AIB1 (Dr Anna Riegel, Georgetown University Medical School, personal communication). The mRNA for AIB-Δ3 was detected at levels higher than normal cells in 7/8 breast cancers (Reiter *et al.*, 2001).

The data in Table 4 show some of the characteristics of AIB1 amplification and expression in breast cancers. Most studies have explored either gene amplification (found in $<10\%$) or mRNA expression (reported in 10–64% of breast tumors). One study reported AIB1 protein expression as being above that seen in normal breast cells in approximately 10% of breast cancers by immunohistochemistry. Protein expression was detected at levels similar to or greater than those seen in normal breast cells in about 60% of ER+ tumors.

The association of AIB1 with ER status is difficult to determine from the small number of studies available. While AIB1 amplification has been associated with ER-positivity (Anzick *et al.*, 1997), increased AIB1 mRNA expression has been associated with ER-negativity (Bouras *et al.*, 2001). Similar proportions of detectable and undetectable AIB1 protein levels ($\sim$65%) were

Table 4 AIB1 amplification and expression in breast cancer (representative studies)

DNA amplification	*mRNA overexpression*	*Protein*	*Study*
10/105 (9.5%)	48/75 (64% relative to normal)	Not reported	Anzick *et al.* (1997)
56/1157 (4%) ER− 10/429 (2.3%) ER+ 45/769 (5.9%)	Not reported	Not reported	Bautista *et al.* (1998)
No data	26/83 (31%) High AIB1: ER+ 11/26 (42%) Low AIB1: ER+ 44/55 (80%)	Not reported	Bouras *et al.* (2001)
Not detected (0%)	3/23 (13%)	Not reported	Glaeser *et al.* (2001)
20/259 (7.7%)	Not reported	Not reported	Cuny *et al.* (2000)
Not reported	Not reported	4/41 (9.8% relative to normal) Present: ER+ 11/16 (69%) Absent: ER+ 12/21 (57%)	List *et al.* (2001)

found in ER+ tumors (12/21 had undetectable expression; 11/16 had detectable expression); no significant correlation between AIB1 and either ER or PR was found (List *et al.*, 2001).

Approximately 10% of all ER+ breast tumors may overexpress wild-type AIB1 protein (List *et al.*, 2001). It remains to be seen if this 10% is primarily comprised of TAM-stimulated tumors, and/or those tumors that exhibit AIB1 gene amplification. One recent study compared AIB1 (western) and erbB2 expression. The 5-year disease-free survival was lower in those tumors expressing high levels of both AIB1 and erbB2 when compared with those expressing high levels of AIB1 and low levels of erbB2. AIB1 and number of positive lymph nodes were also correlated with shorter disease-free survival in TAM-treated compared with untreated patients (Osborne *et al.*, 2003).

Overexpression of AIB1 and AIB1-Δ3 can confer a TAM-stimulated phenotype that should also be estrogen responsive (Dr Anna Riegel, Georgetown University Medical School, personal communication). The proportion of AIB1-overexpressing cells that are dependent upon this activity for survival/proliferation is unknown. The proportion of breast biopsies that respond mitogenically to both TAM and estradiol in short-term culture (4%; see below) suggests that up to one-half of AIB1-overexpressing tumors might be TAM-stimulated. Since these tumors are predicted to retain estrogen responsiveness, and may still synthesize estrogens, many likely retain responsiveness to aromatase inhibitors.

The AIB1-overexpressing phenotype is broadly similar to some MCF-7 TAM-stimulated xenograft models. Since wild-type MCF-7 cells already overexpress AIB1 (Azorsa *et al.*, 2001) and the AIB1-Δ3 (Reiter *et al.*, 2001), it is not surprising that selection against TAM might produce a TAM-stimulated phenotype. Indeed, this phenotype is already present in some MCF-7 cells without TAM selection (Dumont *et al.*, 1996). It remains to be seen whether this model is primarily driven by an overexpression of wild-type AIB1. Since the AIB1-Δ3 was identified in MCF-7 cells and is more potent, this isoform may also contribute to the phenotype of these xenografts and some human breast cancers. Indeed, this variant may prove to be more relevant in a broader context because of its ability to also affect growth factor signaling, an effect that could be important in both ER+ and ER− cells (Reiter *et al.*, 2001).

Clinical relevance of the tamoxifen-stimulated phenotype as an antiestrogen resistance mechanism

Direct evidence of a TAM-stimulated resistance phenotype in breast cancer patients is difficult to find. Indirect evidence may be found from studies that assessed the frequency of a TAM withdrawal response. These responses are evident when a tumor progressing on TAM regresses upon cessation of the TAM therapy. Recently, we completed an extensive review of the literature and found 241 cases in five studies where the authors looked specifically for evidence of TAM withdrawal responses (Clarke *et al.*, 2001b). Responses were assessed by relatively similar criteria and could be combined into three groups: complete response, partial response, and worse than partial response. Evidence was found for only 3/241 complete responses (1.2%) and 13/241 partial responses (5.4%). Over 90% of cases (225/241) experienced a worse than partial response to TAM withdrawal (225/241; 93.4%).

Since breast tumors are highly heterogeneous, the TAM-stimulated population may not be the dominant cell population in most tumors. Thus, elimination of the TAM-dependent/stimulated population may not be sufficient to induce a complete or partial clinical response because the bulk of the tumor is independent of any TAM-induced proliferation. In our evaluation of the literature, disease stabilization was the most common beneficial response to TAM withdrawal. Disease stabilization might indicate tumors that contain populations that are no longer growth-stimulated by TAM and/or a shift in the balance between cell loss/death and proliferation. Whatever the mechanisms, cells in these tumors are clearly not primarily dependent upon TAM for survival, since the great majority of patients (194/241; 80%) experienced disease progression upon TAM withdrawal even when disease stabilization is included as a beneficial response (Clarke *et al.*, 2001b).

These data imply that the majority of tumors in patients that progress on TAM treatment are not progressing because they have acquired a TAM-stimulated phenotype. Indeed, the responses reported for TAM withdrawal may be a mix of several possible mechanisms, including immunologic effects or other mechanisms not directly mediated through ER. Such indirect mechanisms can be largely eliminated in *in vitro* models. A study of 224 human breast cancer biopsies (153 ER+ and 71 ER−) used an *in vitro* approach to measure more directly the frequency of an ER-mediated, TAM- and/or estradiol-stimulated phenotype (Nomura *et al.*, 1990). Primary cultures of breast cancer biopsies were studied for the ability of TAM and estradiol to induce a mitogenic response *in vitro*. Only 11/153 (7%) of ER+ cultures exhibited a mitogenic response to TAM, a proportion surprisingly similar to the proportion (16/241; 6.6.%) of patients estimated to experience either a complete or partial response to TAM withdrawal (Clarke *et al.*, 2001b).

Of interest is the observation that only 6/11 of the TAM-stimulated tumors were also stimulated by estrogen (Nomura *et al.*, 1990). Thus, the TAM- and estradiol-stimulated phenotype, as expressed by some MCF-7 human breast cancer xenografts, reflected only 4% (6/153) of the phenotypes of the ER+ patient biopsies and only 50% of the TAM-stimulated phenotypes.

Together, these data imply that the TAM-stimulated phenotype is only one of several that produce clinical resistance. If up to 20% of initially hormone responsive cases become TAM-stimulated to some degree (estimate includes disease stabilization responses)—by whatever combination of cellular, molecular, and/or immunologic mechanisms this stimulation is conferred—a significant

number of women could be affected. Unfortunately, that still leaves the remaining 80% at risk of acquiring resistance through other mechanisms. From existing evidence, the TAM- and estradiol-stimulated phenotype exhibited by some MCF-7 xenografts may be a minor component of all TAM resistance phenotypes. Clearly, other antiestrogen resistance mechanisms exist, including antiestrogen unresponsiveness, and these remain to be identified and characterized.

Gene networks in estrogen receptor-mediated cell signaling in antiestrogen resistance

ERα expression is both necessary and sufficient to predict responsiveness to antiestrogens in a high proportion of breast tumors. Thus, antiestrogen-induced effects on ERα-mediated signaling are almost certainly of critical importance in effecting clinical responses in many tumors. Nonetheless, we still do not know the genes responsible for signaling to these effects, or whether the effects are primarily to induce cell death, repress cell survival, or a combination of both. As noted above, ER-independent events may also interact with ER-mediated signaling and this may be important in the broader context of a gene network that regulates antiestrogen responsiveness. Thus, estrogens and antiestrogens may differentially affect a gene network that contains some ER-regulated genes (Clarke and Brünner, 1995, 1996). More recently, this concept has been extended to incorporate the likely ability of integrated signals to induce apoptosis while concurrently blocking differentiation and proliferation (Clarke *et al*., 2001c). It is predicted that such a network would be affected by TAM in TAM-stimulated models by signaling through patterns similar to estradiol. In antiestrogen unresponsive cells, signaling through this network may use different signaling patterns and/or exhibit differential regulation/expression of some of the same genes affected by estradiol.

The concept of a network differs from that of a signal transduction pathway in that it requires the integration of several pathways, de-emphasizes the role of well-established single signal transduction pathways, and acknowledges the likelihood that few complex phenotypes are likely to be driven by a single gene/pathway (Clarke *et al*., 2001c). Owing to the plasticity of breast cancer phenotypes, as illustrated by the diversity of endocrine resistance phenotypes (Clarke and Brünner, 1995), the gene network concept seems reasonable. Considering signaling within the constraints of a single, linear pathway may be inappropriate. At best, such an approach is likely to produce an incomplete solution; at worst, it may be misleading.

Delineating the components of a signaling network for estrogens/antiestrogens may not be simple (Clarke and Brünner, 1996). ERs regulate gene expression through direct binding to EREs and direct interactions with other transcription factors including AP-1 and SP-1. The nature of ER activation is affected by ligand structure, and different ligands likely differentially affect the expression and function of the same members of any gene network. For example, raloxifene may regulate gene expression through novel pathways not affected by TAM or ICI 182,780 (Yang *et al*., 1996), and as noted above, antiestrogens differentially affect transcription when bound to ERα compared with ERβ. Regulation of the entire network or key components of the network may also be affected by ER-independent signaling, for example, as intracellular signals are perturbed by tumor–stromal cell interactions. Temporal and spatial organization of signaling components in a network is also critical. The likely complexity of network regulation has been described elsewhere (Clarke *et al*., 2001c).

Accepting the principle of a network is technically demanding because it requires experimental methods to evaluate concurrently the expression of multiple genes and informatic methods capable of integrating expression pattern analyses with functional information. Methods to obtain such high-dimensional data are well established and can be used to explore both the transcriptome and proteome of cells and tumors. However, data analysis methods for exploring gene expression microarray or two-dimensional gel electrophoresis data remain in their infancy and it may be several years before adequate methods become available and widely accepted.

A novel gene expression network in antiestrogen resistance (unresponsiveness)

We have begun to apply both proteome (Skaar *et al*., 1998) and transcriptome analyses (Ellis *et al*., 2002; Gu *et al*., 2002) to breast cancer cell lines, xenografts, and tumors to identify potentially important components of a large signaling network that may contribute to both estrogen independence and acquired antiestrogen resistance. Current informatic methods do not provide an easy way to uncover rapidly and correctly an entire signaling network. However, it should be possible to discover integral components of an overall network and eventually piece together these components to reveal the entire network's structure.

We first identified appropriate cellular models, derived adequate algorithms for data analysis, and began to explore the proteomes by two-dimensional gel electrophoresis and the transcriptomes by serial analysis of gene expression and gene expression microarrays. Remarkably few antiestrogen resistance models are available for study, and almost all are based on the MCF-7 human breast cancer cell line (reviewed in Clarke *et al*., 2001b). MCF-7 xenografts selected against TAM almost exclusively produce a TAM-stimulated phenotype, which may not be representative of the majority of human breast cancers (see below). Thus, we established several E2-independent but responsive breast cancer cell variants with differing antiestrogen response profiles.

MCF-7 cells were first selected for an ability to grow *in vivo* in ovariectomized athymic nude mice. The resulting variant (MCF7/MIII) is estrogen-independent

for growth both in cell culture and as xenografts (Clarke *et al.*, 1989a), but retains responsiveness to antiestrogens; that is, it is estrogen-independent but has an antiestrogen responsive phenotype (Clarke *et al.*, 1989a, b). We further selected these cells *in vivo* and found that repeated *in vivo* estrogen withdrawal, which generated the MCF7/LCC1 variant, did not substantially change the antiestrogen responsiveness of the cells (Brünner *et al.*, 1993a). MCF7/LCC1 cells were then selected *in vitro* for resistance to 4-hydroxytamoxifen. The resulting MCF7/LCC2 cells are TAM-resistant but ICI 182,780 responsive (Brünner *et al.*, 1993b). This phenotype predicted for the subsequent observation that patients responding to TAM, and then acquiring a TAM-resistant phenotype, have a high probability of retaining sensitivity to ICI 182,780 (Howell *et al.*, 1995). In marked contrast, MCF7/LCC1 cells selected for resistance to ICI 182,780 (MCF7/LCC9 variant) acquire resistance to ICI 182,780 and crossresistance to TAM (Brünner *et al.*, 1997). These models represent pharmacologic models of antiestrogen resistance in the context that they no longer respond to the growth inhibitory effects of antiestrogens. Models that reflect a switch to an antiestrogen-stimulated phenotype are described above.

By comparing the proteomes and transcriptomes of several of these MCF7/LCC variants, we have begun to identify what we believe is one component of a larger gene network that may regulate antiestrogen responsiveness. The relevance of this gene subset is already under intensive investigation in functional studies *in vitro* and *in vivo* and for its ability to improve prediction of antiestrogen responsiveness in breast cancer patients.

Candidate genes

The first goal in these studies was to identify differentially expressed genes and proteins that might contribute to acquired estrogen-independent and/or antiestrogen resistance. The data in Table 5 are adapted from our most recent study (Gu *et al.*, 2002) and show the differential regulation of genes we use below to construct one component of a putative antiestrogen responsiveness signaling network. Functional studies of the interactions described in this network are currently in progress.

Table 5 Genes in a putative signaling network

Gene[a]	*Analysis*	*MCF7/LCC1 vs MCF7/LCC9*[b]
EGFR	Microarray	Twofold
EGR-1	Microarray	Threefold
IRF-1	Microarray	Twofold
NFκB	Microarray	0.5-fold
n-ras-related gene	SAGE	0.5-fold
Superoxide dismutase	Microarray	0.5-fold
TNFα	Microarray	Twofold
TNF-R1	Microarray	Twofold
X-box binding protein-1	SAGE	0.25-fold

[a]Links to the UniGene clusters for these and other genes from this study can be found at http://clarkelabs.georgetown.edu/Gu_et_al/Tables.htm. [b]Since the fold differences are relative to MCF7/LCC1 levels, genes upregulated in MCF7/LCC9 cells are expressed as a fraction

Comparing the MCF7/LCC1 and MCF-7 proteomes identified nucleophosmin (NPM) as being associated with estrogen independence (Skaar *et al.*, 1998). NPM is a nucleolar, DNA/RNA-binding phosphoprotein (Wang *et al.*, 1994; Herrera *et al.*, 1995) that, when overexpressed in NIH 3T3 cells, produces a fully transformed phenotype (Kondo *et al.*, 1997). Downregulating NPM delays entry into mitosis (Jiang and Yung, 1999), perhaps reflecting its differential phosphorylation by key kinases: $p34^{cdc2}$ kinase (Peter *et al.*, 1990), CDK2/cyclin E (Tokuyama *et al.*, 2001), and protein kinase C (Beckmann *et al.*, 1992). NPM binds the retinoblastoma protein to induce DNA polymerase α (Tchoudakova *et al.*, 1999) and decreases susceptibility to butyrate-induced apoptosis through inducing telomerase activity (Liu *et al.*, 1999). Overexpression of NPM is seen in colorectal (Nozawa *et al.*, 1996) and prostate cancers (Bocker *et al.*, 1995). NPM is E2-regulated in breast cancer cells (Brankin *et al.*, 1998) and anti-NPM autoantibodies are readily detected in the sera of breast cancer patients (Brankin *et al.*, 1998). NPM blocks the transcriptional activator functions of both YY1 (Inouye and Seto, 1994), which regulates β-casein production in the mammary gland (Raught *et al.*, 1994), and the putative tumor suppressor gene interferon regulatory factor-1 (IRF-1). NPM regulates the stability and activation of p53 (Colombo *et al.*, 2002), implicating its activities in p53-medated effects on apoptosis, and p53 is sequested in the cytosol of TAM-resistant MCF7/LCC2 cells (Lilling *et al.*, 2002).

Exploring the MCF7/LCC1 and MCF7/LCC9 transcriptomes by SAGE identified several differentially expressed genes (Gu *et al.*, 2002). We discuss here only the human X-box binding protein-1 (XBP-1) and the n-ras-related gene. XBP-1 is a member of the ATF/CREB transcription factor family that activates promoters containing CREs (Clauss *et al.*, 1996). During liver regeneration, XBP-1 is associated with increased proliferation and reduced apoptosis (Reimold *et al.*, 2000), implying a survival function that may explain the role of its overexpression in hepatocellular carcinomas (Kishimoto *et al.*, 1998). Expressed within a cluster of genes associated with some ER+ breast tumors (Perou *et al.*, 2000), we have recently begun to explore XBP-1's role in normal and neoplastic breast cells.

The role of the n-ras-related gene is unclear. Ras expression is upregulated in many breast cancers (Clark and Der, 1995) and activates signaling through MAPKs that are also regulated by growth factors implicated in estrogen/antiestrogen responsiveness and mitogenesis (Dickson and Lippman, 1995; Clarke *et al.*, 2001b; Santen *et al.*, 2002). These MAPKs have been implicated in phosphorylating and activating ERs, an effect that could influence antiestrogen responsiveness (Clarke *et al.*, 2001b; Santen *et al.*, 2002). However, some recent studies suggest that MAPK's effects on ER do not

influence antiestrogen responsiveness (Atanaskova *et al.*, 2002).

Exploring the MCF7/LCC1 and MCF7/LCC9 transcriptomes by gene expression microarrays implicated several genes including IRF-1, nuclear factor-κB (NFκB), early growth response gene-1 (EGR-1), epidermal growth factor receptor (EGFR), and both tumor necrosis factor-alpha (TNFα) and its receptor TNF-R1 (Gu *et al.*, 2002). While initially identified as an interferon-induced gene, IRF-1 has now been implicated in regulating several critical cellular functions and is a putative tumor suppressor in some cancers (Tanaka *et al.*, 1994a, b; Yim *et al.*, 1997). IRF-1's tumor suppressor activities may be related to its ability to signal to apoptosis (Tanaka *et al.*, 1994a), which can occur in a p53-dependent or -independent manner (Tamura *et al.*, 1995; Tanaka *et al.*, 1996), with or without induction of $p21^{waf1/cip1}$ (Tanaka *et al.*, 1996) or $p27^{kip1}$ (Moro *et al.*, 2000), and through caspase-1 (Tamura *et al.*, 1995), -7 (Sanceau *et al.*, 2000) -8, (Suk *et al.*, 2001), and/or Fas-ligand (Chow *et al.*, 2000). Potentially related to these activities is the ability of SAPK p38, which is involved in signaling to apoptosis in response to stress, to activate IRF-1/interferon-stimulated reponse element binding (Varley and Dickson, 1999). Consistent with putative tumor suppressor activities, one small immunohistochemical study reports reduced IRF-1 expression in neoplastic *vs* normal human breast tissues (Doherty *et al.*, 2001).

The consequence of NFκB activation is cell context specific (Voegel *et al.*, 1996), but it is generally considered antiapoptotic in most cancer cells. Several aspects of normal mammary gland development appear dependent upon NFκB activity (Clarkson and Watson, 1999), likely reflecting its regulation by both estrogens and growth factors (Nakshatri *et al.*, 1997; Biswas *et al.*, 2000). Elevated NFκB activity arises early during neoplastic transformation in the rat mammary gland (Kim *et al.*, 2000). Widely expressed in human and rat mammary tumors (Sovak *et al.*, 1997; Cogswell *et al.*, 2000), upregulation of NFκB is associated with estrogen independence (Nakshatri *et al.*, 1997; Clarkson and Watson, 1999). NFκB is the only protein known to induce BRCA2 expression (Welcsh and King, 2001). Several excellent reviews on NFκB signaling are available (Bours *et al.*, 2000; Baldwin, 2001; Karin *et al.*, 2002).

EGR-1 is a transcription factor with proapoptotic activity (Das *et al.*, 2000) and is downregulated in DMBA-induced mammary adenocarcinomas in rats and mouse and human breast cancer cells (Huang *et al.*, 1997). c-myc is a major regulator of breast cancer proliferation and survival (Liao and Dickson, 2000) and is among the genes downregulated by EGR-1 (Hoffman *et al.*, 2002). EGR-1 also blocks NFκB function (Chapman and Perkins, 2000) and can stimulate apoptosis through cooperation with $p21^{waf1/cip1}$ and transactivation of p53 (Liu *et al.*, 1998). Superoxide dismutase (SOD) expression is increased in MCF7/LCC9 cells (Gu *et al.*, 2002) and in TAM-stimulated MCF-7 xenografts (Schiff *et al.*, 2000); SOD overexpression was previously implicated in resistance to TNFα (Zyad *et al.*, 1994). A TNFα-mediated pathway for signaling to apoptosis occurs in MCF-7 cells (Burow *et al.*, 1998; Egeblad and Jaattela, 2000), and measuring serum TNF concentrations may be a useful prognostic marker in breast cancer patients (Sheen-Chen *et al.*, 1997). Furthermore, IRF-1 expression is induced by TNFα in some cells (Mori *et al.*, 1999).

One component of a gene network

Using the data from our proteome and transcriptome studies and from other published studies, we have begun to construct a gene expression network for signaling in antiestrogen responsiveness (Figure 3). Studying a variant that is crossresistant to triphenylethylenes and steroidal antiestrogens (MCF7/LCC9) provided the opportunity to identify more broadly based resistance signaling than might be obtained from a study of TAM-only resistance (e.g., MCF7/LCC2 phenotype). The apparent consistency of the interactions among the

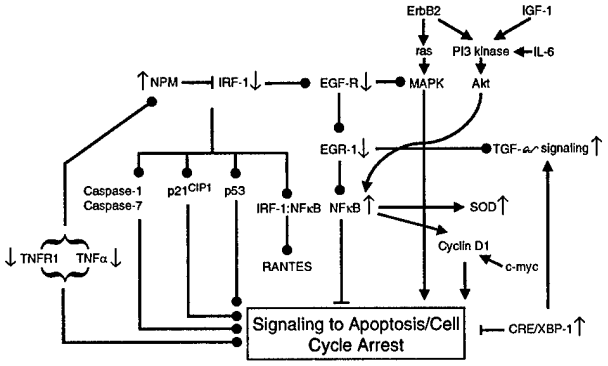

Figure 3 Part of a putative gene expression network constructed from the genes differentially expressed in MCF7/LCC9 cells (TAM and ICI 182,780 crossresistant) and their sensitive MCF7/LCC1 parent cells. Candidate genes from other studies are also incorporated into the network. Arrows represent those genes with altered expression, and the consequences of these changes are represented in the context of an antiestrogen-resistant phenotype. For example, the low levels of IRF-1 in MCF7/LCC9 cells are unable to induce EGFR, which remains low in these cells. Redundancy is evident; for example, the upregulation of NFκB and ras may compensate for low EGFR expression because they signal downstream of the EGFR's kinase activity. Signaling through this network component is expected to be different between sensitive and resistant cells and likely also different among some populations with the same phenotype. For example, not all resistant cells need to modify gene expression in the same pattern as apparently adopted by MCF7/LCC9 cells. Since ER-mediated effects are critical in antiestrogen-induced signals in sensitive cells, these cells may signal through the network component primarily comprising ER-regulated genes. While the interactions in this figure are consistent with published data, the network as represented is not intended to be complete and the regulation of some genes may be more complex than alluded to here. As we further evaluate signaling in these cells, we may identify additional components of this network. {} = receptor–ligand complex; ↑ = expression is increased; ↓ = expression is reduced; other arrows show direction of signal transduction; ⊥ = inhibition of indicated gene/function; —● = inability to induce substantially next signal or influence next event due to low/reduced expression/activity

relatively few genes incorporated into our network component is surprising. EGF-R induces expression of EGR-1 (Tsai *et al.*, 2000), and expression of both genes is lower in MCF7/LCC9 cells (Gu *et al.*, 2002). Since EGR-1 inhibits NFκB function (Chapman and Perkins, 2000), its low expression may contribute to the increased NFκB activity in these cells (Gu *et al.*, 2002). IRF-1 induces EGF-R mRNA (Rubinstein *et al.*, 1998), and IRF-1 levels are lower in MCF7/LCC9 cells (Gu *et al.*, 2002). IRF-1 is induced by TNFα/TNF-R1 (Mori *et al.*, 1999), both of which are also concurrently downregulated in MCF7/ LCC9 cells, perhaps explaining their lower IRF-1 levels. IRF-1 can act as a tumor suppressor and signal to apoptosis through both p53-dependent and -independent pathways (Taniguchi, 1997). These observations may reflect IRF-1's ability to affect caspase activity, since caspase activation and induction of apoptosis are implicated in affecting antiestrogen responsiveness (Mandlekar *et al.*, 2000a, b). Overexpression of caspase-1, which regulates apoptosis in normal mammary epithelial cells (Boudreau *et al.*, 1995), is known to be lethal in MCF-7 cells (Keane *et al.*, 1996). In these models, signaling through caspase-3 is unlikely because the gene is truncated in MCF-7 cells (Friedrich *et al.*, 2001); signaling through caspase-7 may dominate.

Interferons (IFNs) and TNF act synergistically to induce gene expression, an effect that appears driven by protein–protein interactions between IRF-1 and NFκB (Drew *et al.*, 1995; Neish *et al.*, 1995). IRF-1 can induce degradation of IκBα in some cells (Kirchoff *et al.*, 1999). IRF-1 : NFκB heterodimers affect expression of the ATF-2/jun (Escalante *et al.*, 1998), RANTES (Lee *et al.*, 2000), VCAM-1 (Neish *et al.*, 1995), IL-6 (Sanceau *et al.*, 1995), and MHC class 1 genes (Drew *et al.*, 1995). Altered AP-1 expression (includes jun) is implicated in the TAM-stimulated antiestrogen resistance phenotype (Schiff *et al.*, 2000), RANTES expression correlates with a poor prognosis (Luboshits *et al.*, 1999), VCAM-1 is involved in angiogenesis and metastasis in breast tumors (Byrne *et al.*, 2000), and autocrine production of IL-6 is associated with drug resistance in breast cancer cells (Conze *et al.*, 2001).

Unlike IRF-1, NPM expression is increased in MCF7/LCC9 cells compared with MCF7/LCC1 cells. NPM can function as an oncogene, its overexpression fully transforming NIH 3T3 cells in an assay for oncogenic potential (Kondo *et al.*, 1997). Levels of autoantibodies to NPM increase in patients 6 months prior to recurrence. Consistent with an antiestrogenic regulation of NPM, the levels of NPM autoantibodies are lower in breast cancer patients who received TAM (Brankin *et al.*, 1998). Concurrent upregulation of NPM and downregulation of IRF-1 suggest a novel signaling pathway in antiestrogen resistance. Both are estrogen-regulated genes in MCF-7 cells, IRF-1 expression being suppressed and that of NPM being induced (Skaar *et al.*, 1998, 2000). Through its direct binding to IRF-1, NPM inhibits the transcription regulatory activities of IRF-1 (Kondo *et al.*, 1997). Overexpression of NPM may eliminate the remaining IRF-1 activity, blocking its ability to initiate an apoptotic caspase cascade, and/or induce $p21^{waf/1cip1}$ (Coccia *et al.*, 2000) and cooperate with p53 in signaling to growth arrest and apoptosis (Tanaka *et al.*, 1994a, 1996).

XBP-1 acts through its ability to regulate genes containing CRE in their promoters (Clauss *et al.*, 1996). A cAMP-dependent pathway that inhibits IRF-1 transactivation has been described (Delgado *et al.*, 1999); XBP-1 activation of this pathway could suppress further the already low IRF-1 activity in some antiestrogen-resistant cells.

N-ras-induced signaling may also be important and implies an upregulation of ras-induced signaling in resistant cells. Such increased signaling may partly abrogate the need for growth factor-induced signaling through autocrine, paracrine, or intracrine stimulation (Clarke *et al.*, 2001b) because increased ras activation is downstream of several growth factor receptors implicated in breast cancer (Santen *et al.*, 2002). For example, cells may be capable of surviving when EGFR levels are reduced (Table 5) because loss of EGFR signaling is compensated by a downstream upregulation of ras-mediated signaling. Low IRF-1 expression may also contribute to the effects of ras signaling because IRF-1 induces lysyl oxidase (Sers *et al.*, 2002), which is implicated in reversing ras-induced malignant transformation (Contente *et al.*, 1999; Nozawa *et al.*, 1999).

Some of the genes we found have been implicated in antiestrogen resistance in other studies, most notable being EGF-R (Nicholson *et al.*, 2001) and its family member c-erbB2 (Kurokawa *et al.*, 2000; Welch and Clarke, 2002; Konecny *et al.*, 2003). AKT (Perez-Tenorio and Stal, 2002), c-myc (Carroll *et al.*, 2002), cyclin D1 (Varma and Conrad, 2002), p53, $p21^{waf1/cip1}$ (Fattman *et al.*, 1998), and AP-1 (Schiff *et al.*, 2000) may also contribute to antiestrogen responsiveness. We have incorporated some of this knowledge into the network in Figure 3, particularly where these genes may interact with those identified in our models. Several genes are thought to be downstream of signaling from growth factor receptors implicated in either phosphorylating/activating ER and/or inducing mitogenesis and affecting antiestrogen responsiveness (Chan *et al.*, 2001; Varma and Conrad, 2002). For example, the type I insulin-like growth factor receptor and c-erbB2 can activate AKT, which is often upstream of NFκB (Martin *et al.*, 2000). Several growth factors activate MAPK signaling to mitogenesis and signal through activation of ER. For simplicity, we have not shown all of these possible interactions in Figure 3.

Functional studies

We acknowledge that the gene network component in Figure 3 is somewhat speculative. Furthermore, it is unlikely to be regulated in the same way in TAM-stimulated models that perceive TAM as an estrogen. For example, in TAM-stimulated models, key network components could be perturbed in the same manner as expected with estradiol treatment.

One approach to assessing the likely validity of selected genes in our network component is to explore their functional activities and abilities to affect antiestrogen responsiveness in experimental models. We have begun several studies to further assess the likely functional relevance of our observations and support the gene network component in Figure 3. Transcriptional activation of XBP-1 and NFκB was studied using established promoter–reporter assays (CRE promoter–reporter assay for XBP-1). As predicted in the transcriptome analyses, increased basal transcription of both promoters was observed. Further studies showed that the ability of ICI 182,780 to inhibit NFκB activation is lost in the resistant cells. Preliminary data from our laboratory imply that the ability of antiestrogens to induce IRF-1 is also lost in resistant cells (Bouker *et al.*, 2002). Consistent with our earlier hypotheses (Clarke and Lippman, 1992), these data show significant changes in the endocrine regulation of some ER-regulated genes. We found no evidence for endocrine regulation of CRE activation in either responsive or resistant cells. However, resistant cells exhibit a significant fourfold increase in CRE activation, reflecting the fourfold increase in its expression predicted from the SAGE study. These observations suggest at least some general resistance mechanisms: an overexpression and loss of endocrine regulation of some genes that are ER-regulated in responsive cells, a downregulation and loss of endocrine regulation of some genes that are ER-regulated in responsive cells, and an upregulation of some endocrine unresponsive genes.

To study functional relevance further, the sensitivity of our variants to inhibition of NFκB activation by parthenolide was explored. Parthenolide, which is currently in early clinical trials, binds NFκB in a highly stereospecific manner (Garcia-Pineres *et al.*, 2001) and inhibits the IκB kinase repressor of NFκB (Hehner *et al.*, 1999; Patel *et al.*, 2000). We would expect that, if NFκB is providing a survival function, MCF7/LCC9 cells might be more dependent upon this activity. Indeed, MCF7/LCC9 cells are significantly more sensitive to growth inhibition by parthenolide than their MCF7/LCC1 parental cells (Gu *et al.*, 2002). Thus, some cells may survive antiestrogen exposure by upregulating estrogen-regulated survival factor(s) concurrent with the loss of their ER-mediated regulation. While we first need to confirm and extend these observations, parthenolide may prove useful in combination with Faslodex or other antiestrogens to either increase responsiveness and/or delay the appearance of resistant disease. Functional studies into the activities of the other genes in this network and investigations into their power to better predict antiestrogen responsiveness in patients are in progress.

Conclusions and future prospects

Acquired antiestrogen resistance likely comprises both true antiestrogen unresponsiveness (the major phenotype) and antiestrogen-stimulated growth (probably a minor phenotype). Several resistance mechanisms exist and, with the exception of loss of ER expression, these mechanisms may not be driven by a single gene or single signaling pathway. Consequently, we continue to develop the concept that an integrated gene network exists that allows cells a significant degree of plasticity in how they signal through this network (Clarke and Brünner, 1995, 1996; Clarke *et al.*, 2001c). More recently, we have begun to identify candidate genes in one component of this network and to explore their likely functional relevance in experimental models and ability to predict patient outcome. As we and others explore the transcriptomes and proteomes of experimental models and patient samples, additional components of this network may become apparent. Ultimately, understanding how breast cancer cells coordinate a response to antiestrogens, and overcome the growth inhibitory nature of the resulting signaling, may lead to better treatments and more powerful predictors of clinical response.

Some dietary components can modify the ability of TAM to inhibit the growth of ER+ and perhaps also ER− breast cancer cells. These dietary components might be those that alone are believed to affect recurrence of breast cancer. However, when consumed in combination with TAM, various dietary components could either potentiate or inhibit TAM's actions. Examples of unexpected findings are the studies of Ju *et al.* (2002) and Depypere *et al.* (2000), who showed that genistein or tangeretin prevents TAM from inhibiting growth of malignant breast cells. Currently, only a few published studies have examined the impact of nutrition on TAM's therapeutic effects, and it is likely that other dietary factors can modify TAM's ability to inhibit breast cancer growth.

The clinical use of antiestrogens, and TAM in particular, may change in the future. Data from some recent studies suggest that the current generation of aromatase inhibitors may be more effective than antiestrogens as first-line endocrine treatment for ER+ metastatic breast cancer and as adjuvant therapy for ER+ breast primaries (Buzdar and Howell, 2001; Ellis *et al.*, 2001). Nonetheless, the American Society of Clinical Oncology's Technology Assessment Working Group continues to recommend 5 years of adjuvant TAM as the standard therapy for women with ER+ breast cancer (Winer *et al.*, 2002). In terms of chemoprevention, the recommendations include the use of TAM *vs* participation in a clinical trial that involves the administration of raloxifene, any aromatase inhibitor, or any retinoid only within the context of chemoprevention (Chlebowski *et al.*, 2002).

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Review Article

erbB2 expression and drug resistance in cancer

This review will summarise the current data regarding the role of erbB2 as a molecular marker for therapeutic intervention and drug resistance.

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Introduction

Identification of molecular markers that could serve as accurate predictors of response to specific cytotoxic chemotherapies would be useful in targeting such therapies at individual patients. One potential molecular marker is erbB2, a transmembrane receptor tyrosine kinase and member of the EGFR superfamily, which has been implicated in the generation and/or progression of a number of different carcinomas, notably tumours of the breast.[1–4] Increased expression of erbB2 is frequently associated with more aggressive cancers and has been implicated in conferring resistance to some drugs.

Consequently, several erbB2-specific therapeutic agents have been developed to target tumours that specifically overexpress this protein. Herceptin™ (trastuzumab), an anti-erbB2 monoclonal antibody and the first erbB2 approach to be approved for use, has shown activity in some breast cancers and may improve response to specific cytotoxic drugs.[5,6] The activity of Herceptin™ has thus far been attributed to blocking the mitogenic growth signalling driven by erbB2 and/or eliciting an antitumour immune response.[7]

The mechanisms by which erbB2 promote drug resistance have not been established. Nor has the validity of using erbB2 expression to predict potential drug resistance been proven.

This review summarises and discusses the role of erbB2 as a molecular marker for therapeutic intervention and drug resistance in cancer.

Background

Increased expression of erbB2 has been observed in many solid tumours (table 1), including breast,[8,9] prostate,[10,11] ovarian,[12] colorectal,[13,14] endometrial,[15] and non-small-cell lung cancers.[16–18] In most cases, increased expression of erbB2 is associated with poor prognosis and is correlated with decreased relapse-free and overall survival.[10,15,19,20] An exception may be colorectal tumours, which do not consistently demonstrate

High expression of erbB2 is associated with more aggressive cancers

The mechanism by which erbB2 promotes drug resistance is unknown

Table 1. Increased levels of erbB2 expression in selected solid tumours

Tumour	Observed increased erbB2 expression	References
Breast	25–30%	Slamon et al.[8,9]
Colorectal	35–38%	Lazaris et al.[13] Kluftinger et al.[14]
Endometrial	17%	Rolitsky et al.[15]
Non-small-cell lung	29–65% (varies with stage)	Han et al.[16] Graziano et al.[17] Kristiansen et al.[18]
Ovarian	46%	Leng et al.[12]
Prostate	36–64%	Morote et al.[10] Fox et al.[11]

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a clear relationship between erbB2 expression and prognosis.[21,22]

Despite the frequent association of erbB2 expression with more aggressive tumours, the precise mechanisms involving tumour progression are not clearly defined. However, several possibilities have been proposed that may either work in concert or function independently in a tissue-specific manner. For example, erbB2 up-regulation can enhance the activation of Akt, a serine–threonine kinase involved in antiapoptotic signalling.[23] In breast tumour biopsies, increased erbB2 expression correlates with enhanced Akt-mediated activation of NF-κB, a transcription factor known to increase the production of cell survival proteins.[24–26] erbB2 levels have also been correlated with both enhanced Akt expression and Akt-mediated resistance to apoptosis induced by either UV irradiation or hypoxia in breast cancer cells.[24] Similar findings have also been demonstrated in prostate,[27] ovarian[28] and non-small-cell lung cancer cell lines.[29]

erbB2 and drug resistance

Several signalling pathways affecting apoptosis may be influenced by erbB2 activity. For example, elevated erbB2 expression correlates with greater resistance to tumour necrosis factor (TNF)-α-induced apoptosis in several *in vitro* models, including breast and ovarian cancer cells,[24] NIH 3T3 cells[30] and cervical carcinoma cells.[31]

erbB2-mediated resistance appears to be dependent upon Akt activation.[28] Conversely, inhibition of erbB2 signalling enhances TNF-α-mediated apoptosis in breast, ovarian[28] and lung cancer cells.[32]

Other molecular mechanisms that may contribute to erbB2-mediated drug resistance include disruption of cell cycle checkpoint proteins, increased signalling through growth-promoting pathways, and modulation of oestrogen receptor (ER) function. The likely role(s) of these effects in drug resistance are described below, beginning with the possible effects of erbB2 expression on response to anti-oestrogens.

Although erbB2 expression has been widely implicated in affecting response to various antineoplastic drugs, the typical association of erbB2 overexpression with poor clinical outcome complicates the assessment of its role in drug resistance in some studies. Tumours with a poor prognosis may have a poor clinical outcome irrespective of treatment, reflecting their biological progression rather than any specific drug resistance. When examined in breast cancer models, such as normal mammary epithelial cells or human breast cancer cell lines coaxed to overexpress erbB2 by transfection,[33] erbB2 overexpression does not confer drug resistance. However, co-expression of erbB2 with other EGFR family members can produce resistance to several chemotherapeutic agents commonly used to treat breast cancer.[34] These observations suggest that cellular context (the pattern of other genes/proteins expressed within a cell[35]) can significantly affect erbB2 signalling and drug responsiveness.

erbB2-mediated resistance to tamoxifen

Resistance to the triphenylethylene anti-oestrogen tamoxifen has been correlated with erbB2 expression in several *in vitro* studies,[36–39] but the mechanisms are unclear.[35] Protein–protein interactions between erbB2 and ER have been described in cell membranes and may protect breast cancer cells from tamoxifen-induced apoptosis by preventing tamoxifen–ER interactions.[36] Overexpression of erbB2 in MCF-7 breast cancer cells prevents tamoxifen-induced apoptosis, apparently by up-regulating the antiapoptotic bcl-2 and bcl-xL proteins.[37] erbB2 signalling via MAP kinase activation has also been proposed as a mechanism for tamoxifen resistance in breast cancer cells.[38]

Overexpression of erbB2 correlates with greater resistance to TNF-α-induced apoptosis

The ability of erbB2 to induce Akt-mediated NF-κB signalling to promote cell survival implicates this pathway as another mechanism for anti-oestrogen resistance. Recently, indirect evidence in support of this hypothesis was obtained using gene-expression microarray analysis of anti-oestrogen resistant cells, and increased NF-κB expression in cells surviving treatment with the anti-oestrogen Faslodex™ (fulvestrant) was reported.[40] Furthermore, anti-oestrogen-resistant cells have up-regulated NF-κB transcriptional activation, marked by a lack of anti-oestrogen regulation of this activation, and are more sensitive to inhibition of NF-κB activity by the small-molecule inhibitor parthenolide.[40] Since NF-κB is downstream of both erbB2 and Akt, some tumours may become resistant without increased activity of either upstream component (fig. 1). Thus, cellular context may be a key determinant in how erbB2 signals in anti-oestrogen-resistant breast cancers. It remains to be seen whether including measurements of erbB2, Akt and NF-κB will improve the ability to predict anti-oestrogen responsiveness in breast cancer.

Tumours that overexpress erbB2 exhibit a decreased response to tamoxifen

Several clinical studies have also shown that tumours overexpressing erbB2 exhibit a decreased response to tamoxifen when compared to tumours without erbB2 overexpression.[41–43] However, some studies are difficult to interpret because erbB2 overexpression is often associated with ER negativity and ER-negative tumours rarely respond to anti-oestrogens.[35]

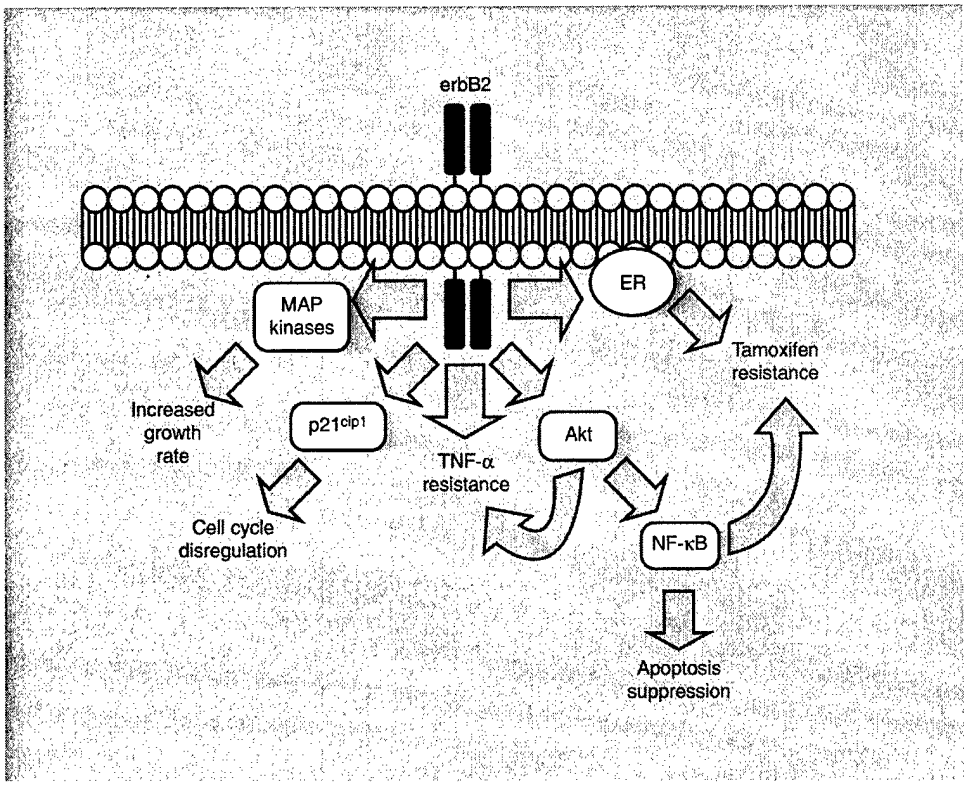

Figure 1. erbB2 signalling pathways and their implication in conferring resistance to antineoplastic drugs.

Herceptin™ in combination with either paclitaxel or docetaxel is effective in breast cancer trials

High expression of erbB2 in breast tumours generally correlates with increased response to anthracycline-based regimens

Overexpression of erbB2 might serve as a biomarker for predicting anthracycline responsiveness in patients

The combination of tamoxifen and Herceptin™ is more effective in treating erbB2-positive tumours than either agent alone.[44] While this is pharmacological antagonism,[45] blocking erbB2 activity during the administration of tamoxifen could still provide clinical benefit.

erbB2 and resistance to taxoids

Data from several *in vitro* studies indicate that erbB2 may contribute to paclitaxel resistance in breast and head and neck cancer cells.[46–48] The opposite effect was reported in one study in ovarian cancer cells, where increased erbB2 expression correlated with increased sensitivity to paclitaxel.[49] Whether this latter result truly reflects a different relationship between erbB2 expression and paclitaxel resistance in ovarian tumours, in comparison to other solid tumours, requires further study.

Two mechanisms of paclitaxel resistance have been attributed to erbB2 signalling, other than simply blocking direct signalling to apoptosis. First, erbB2 disrupts paclitaxel-induced cell cycle arrest at the G2/M checkpoint, an effect that normally leads to apoptosis through the involvement of the serine-threonine kinase $p34^{cdc2}$,[50] erbB2 signalling has been associated with increased expression of $p21^{cip1}$, which inhibits the function of $p34^{cdc2}$ and allows cells to bypass the G2/M checkpoint and avoid paclitaxel-induced apoptosis.[51,52] Second, paclitaxel disrupts the cell cycle by interfering with the microtubule functions associated with mitosis. NIH-3T3 cells genetically engineered to overexpress erbB2 show alterations in β-tubulin isotype expression patterns associated with paclitaxel resistance.[53]

Currently, combinations of taxoids with anti-erbB2 therapeutics are being tested in the clinical setting. Data available from phase II trials in breast cancer patients, in whom the use of Herceptin™ is appropriate, indicate that a combination of Herceptin™ with either paclitaxel or docetaxel is both effective and tolerable.[54–56]

While these early studies suggest that combinations of Herceptin™ and taxoids are potentially more effective than other drug combinations, further study is needed before firm conclusions can be drawn.[54,57,58]

Anthracycline responsiveness and erbB2 expression

Like the taxoids, the anthracylines, particularly Adriamycin™ (doxorubicin), are among the most

effective single agents for breast cancer and are commonly used in combination first-line therapy. In contrast to the taxoids, high levels of erbB2 expression in breast tumours generally correlate with increased response to anthracycline-based regimens,[59-62] although exceptions have been noted.[63,64]

Anthracylines inhibit topoisomerase II activity, and the erbB2 and topoisomerase IIα genes, which are located near each other on chromosome 17, are often co-expressed. A functional interaction between erbB2 and topoisomerase IIα has been reported, where erbB2 increases topoisomerase II activity.[53] Thus, some tumours that overexpress erbB2, independent of gene amplification, may also express sufficient levels of topoisomerase II to exhibit increased sensitivity to anthracyclines. Some investigators have suggested that overexpression of erbB2 might serve as a biomarker for predicting anthracycline responsiveness in patients, but this requires further study.[65]

erbB2 and EGFR

The erbB2 signalling network is an attractive molecular target in breast cancer, especially in ER-negative disease. Although inhibition of erbB2 results in tumour regression in a cohort of patients with metastatic disease, it is less clear whether targeting other receptors in this signalling network would be of therapeutic benefit.

Aberrant EGFR and erbB2 signalling has been causally associated with enhanced breast cancer cell proliferation and shorter survival in patients with mammary carcinomas.[8,66,67] Also, studies with breast cancer cell lines and human tumours have demonstrated constitutive phosphorylation of erbB2.[68,69] The reasons for this constitutive activation are not clear but one possibility includes co-expression of ligand-activated EGFR resulting in transactivation of the erbB2 tyrosine kinase. Indeed, in cells that co-express erbB2, ligand-activated EGFR preferentially recruits erbB2 into a heterodimeric complex that exhibits an increased rate of recycling, stability, and signalling potency compared to EGFR homodimers.[70,71]

The recent work by Shou et al. highlights the important interaction between EGFR and erbB2.[72] The authors examined the cross-talk between the ER and the EGFR/erbB2-receptor family by examining the ER-positive (MCF-7) and tamoxifen-resistant erbB2-overexpressing (HER2-18) breast cancer cell lines. In both cell lines, ZD1839 ('Iressa') inhibited ER, EGFR and erbB2 phosphorylation induced by epidermal growth factor and heregulin, but not that by oestrogen. Interestingly, in the tamoxifen-resistant HER2-18 cells, ZD1839 completely inhibited both oestrogen- and tamoxifen-induced phosphorylation and activation of erbB2.

Following on from this observation, long-term studies to investigate whether ZD1839 treatment can delay or prevent development of resistance to various endocrine therapies are in progress.

Another intriguing finding from studying breast cancer cells is that high expression levels of erbB2, even in the presence of a low number of EGFR, are exquisitely sensitive to ZD1839. Using a panel of breast cancer cell lines representative of the entire spectrum of EGFR and erbB2 alterations found in breast cancer patients, Campiglio et al.[73] showed that growth inhibition of these cell lines demonstrates that sensitivity to ZD1839 did not depend on the level of EGFR expression.

If receptor cooperativity is in fact operational in breast cancers, interruption of EGFR function with EGFR-specific tyrosine kinase inhibitors, such as ZD1839 may disrupt EGFR-erbB2 cross-talk and result in erbB2 inactivation as well. This inactivation of erbB2 through the inhibition of EGFR may also increase the antitumour effect of Herceptin™.

ZD1839 completely inhibited both oestrogen- and tamoxifen-induced activation of erbB2 in tamoxifen-resistant HER2-18 cells

Summary

The association between erbB2 expression and sensitivity of the tumour/cancer cells to chemotherapy has been widely studied, particularly in breast cancer. Currently, data suggest that high levels of erbB2 confer some degree of resistance to taxoids but may sensitise cells to anthracyclines. Adjuvant chemotherapy regimens using other cytotoxic drugs (e.g. methotrexate, 5-fluorouracil, cisplatin) have also demonstrated reduced efficacy in high erbB2 expressing tumours in comparison to those with low erbB2 expression.[74-77] However, others have found no link between erbB2 expression and response to adjuvant chemotherapy in some breast tumours.[78-81] More recently, investigators have begun to study combinations of Herceptin™ with other chemotherapeutic agents, such as gemcitabine in breast cancer[69] and estramustine in prostate cancer.[82] Several such combinations show promise and may become more widely accepted in the near future.

The association between erbB2 expression and poor prognosis is well established, making erbB2 a useful prognostic marker in some cancers. Evidence clearly suggests that overexpression of erbB2 may also be a useful predictor of responsiveness to specific drugs in some tumours. However, the complexity of cell signalling and the importance of cellular context may dictate thatsolely measuring erbB2 expression may not be sufficiently discriminative. It

may be necessary to identify a series of additional genes which, when information on their expression patterns are combined, will produce a sufficiently powerful predictor of drug-specific responsiveness.

The mechanisms by which erbB2 expression affects drug responsiveness may be more complex than currently appreciated and may require a better understanding of cellular context and the factors that affect erbB2 signalling to proliferation/ cell survival. However, gene-expression microarray and proteomic technologies have the power to better define cellular context and identify genes/ proteins that can modify erbB2 signalling. Some of these genes may even provide new targets for drug development. Since erbB2 sensitises cells to some drugs while conferring resistance to others, measuring erbB2 expression and those genes that affect its downstream signalling could enable the targeting of specific therapies to individual tumours.

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Block principal component analysis with application to gene microarray data classification

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SUMMARY

We propose a block principal component analysis method for extracting information from a database with a large number of variables and a relatively small number of subjects, such as a microarray gene expression database. This new procedure has the advantage of computational simplicity, and theory and numerical results demonstrate it to be as efficient as the ordinary principal component analysis when used for dimension reduction, variable selection and data visualization and classification. The method is illustrated with the well-known National Cancer Institute database of 60 human cancer cell lines data (NCI60) of gene microarray expressions, in the context of classification of cancer cell lines. Copyright © 2002 John Wiley & Sons, Ltd.

KEY WORDS: principal component analysis; grouping of variables; similarity; gene expression; microarray data analysis

1. INTRODUCTION

Principal component analysis is one of the most common techniques of exploratory multivariate data analysis. It is a method of transforming a set of p *correlated* variables $\mathbf{x} = (x_1, x_2, \ldots, x_p)$ to a set of p *uncorrelated* variables $\mathbf{y} = (y_1, y_2, \ldots, y_p)$ that are linear functions of the x's, referred to as p principal components of $\mathbf{x}$, such that the variances of the y's are in descending order with respect to the variation among the x's. Usually the first several components explain most of the variation among the x's. In addition to many other applications, principal component analysis has been shown to be a useful tool in reducing data dimension and extracting information, in seeking important regressors in regression analysis, and in effectively visualizing and clustering subjects, when measurements on a large number of variables are collected from each subject. The book by Jolliffe [1] provides excellent reading on this topic, although other textbooks on multivariate data analysis do also (for

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example, references [2] and [3]). Recently, principal component analysis has found application in the analysis of microarray gene expressions [4], a growing technology in human genome studies [5, 6].

When dealing with an extremely large number of variables (for example, 500 or more), deriving principal components can be computationally intensive, since it involves finding the eigenvectors (and eigenvalues) of a matrix with large dimensions. Moreover, a linear combination of such a large number of variables becomes less meaningful to the investigators since the high dimensionality makes it hard to extract useful information and to interpret the combination. In one microarray technology, cDNA clone inserts are printed onto a glass slide and then hybridized to two differentially fluorescently labelled probes. The final gene expression profile contains fluorescent intensities and ratio information of many hundreds or thousands of genes. If one intends to apply principal component analysis directly to extract gene expression information for these genes from a certain group of subjects, then one has to deal with a matrix with huge dimensions.

In dealing with such high dimensional data, we propose to perform the principal component analysis in a '*stratified*' way. We first group the original variables into several '*blocks*' of variables, in the sense that each block contains variables (genes in the microarray experiments) that are similar; variables from the same block are more correlated than variables from different blocks. We then perform principal component analysis within each block and obtain a small number of variance-dominating principal components. Combining these principal components obtained from each block forms a new database from which we can then extract information by performing a new principal component analysis. We term this procedure as '*block principal component analysis*'. Dominating principal components obtained from the final stage can then be used in various data exploratory analyses such as clustering and visualization.

The proposed 'block principal component analysis' method also enables us to reduce the number of variables effectively. Within each block, when principal component analysis is conducted and dominating linear combinations of variables are examined, only those variables that have relatively large coefficients are retained. We will examine this variable selection procedure in detail using the gene microarray example.

After a brief review of the mathematical derivation of principal components and their applications in Section 2, we introduce in Section 3 the method of 'block principal component analysis'. In Section 4, we investigate the efficiency of block principal components in the reduction of data dimension with respect to the amount of variance explained. It is shown that the proposed procedure can be as efficient as the ordinary principal component analysis. We then discuss the selection of informative variables using block principal component analysis. In Section 5 we apply the method to the problem of classification of microarray data from the well-known National Cancer Institute database of 60 human cancer cell lines (NCI60), each of which has gene microarray expression of more than 1000 genes [7]. Some discussion is given in Section 6.

2. PRINCIPAL COMPONENTS

We start with a brief mathematical derivation of principal components. More details can be found in references [1] or [2] and [3]. Throughout, vectors are viewed as column vectors, and $\mathbf{A}'$ is the transpose of a matrix $\mathbf{A}$.

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Consider a p-variate random vector $\mathbf{X}$ with mean vector μ and positive definite covariance matrix $\mathbf{\Sigma}$. Let $\lambda_1 \geqslant \lambda_2 \geqslant \cdots \lambda_p (>0)$ be the eigenvalues of $\mathbf{\Sigma}$ and let $\mathbf{W} = (\mathbf{w}_1, \ldots, \mathbf{w}_p)$ be a $p \times p$ orthogonal matrix such that

$$\mathbf{W}'\mathbf{\Sigma}\mathbf{W} = \mathbf{\Lambda} = \text{diag}(\lambda_1, \ldots, \lambda_p) \tag{1}$$

so that $\mathbf{w}_i$ is an eigenvector of $\mathbf{\Sigma}$ corresponding to the eigenvalue λ_i. Now put $\mathbf{U} = \mathbf{W}'\mathbf{X} = (U_1, \ldots, U_p)'$; then $\text{cov}(\mathbf{U}) = \mathbf{\Lambda}$, so that $U_1, \ldots, U_p$ are all uncorrelated, and $\text{var}(U_i) = \lambda_i$, $i = 1, \ldots, p$. The linear components $U_1, \ldots, U_p$ are called principal components of $\mathbf{X}$. The first principal component is $U_1 = \mathbf{w}_1'\mathbf{X}$ and its variance is λ_1; the second principal component is $U_2 = \mathbf{w}_2'\mathbf{X}$ with variance λ_2; and so on. These p principal components have the following key property. The first principal component U_1 is the normalized (unit length) linear combination of the components of $\mathbf{X}$ with the largest variance, and its maximum variance is λ_1; then out of all normalized linear combinations of the components of X which are uncorrelated with U_1, the second principal component U_2 has maximum variance λ_2. In general, the kth principal component U_k has maximum variance λ_k, among all normalized linear combinations of the components of $\mathbf{X}$ which are uncorrelated with $U_1, \ldots, U_{k-1}$.

Very often these principal components are referred to as population principal components. In practice $\mathbf{\Sigma}$ is not known and has to be estimated from the sample, yielding the sample principal components. We do not distinguish these two definitions here.

Once the p principal components are derived, then we can conduct various statistical analyses using only the first $q (< p)$ principal components which account for most of the variance of $\mathbf{X}$. For example, we can plot the first two (three) principal components in a two- (three-) dimensional space to seek interesting patterns among the data, or perform clustering analysis on subjects in order to search for clusters among the data. We can also use these leading principal components as regressors in a regression analysis to find prognostic factors for clinical outcomes (for example, drug response or resistance). See reference [1] for various other applications of principal component analysis.

Derivation of principal components involves computation of eigenvalues and eigenvectors of the $p \times p$ matrix $\mathbf{\Sigma}$ (or its sample estimate). When p is very large, the computation will become extremely extensive. Moreover, it is always the interest of the investigators to examine the first several leading principal components in order to find useful information. With a linear combination of a large number of variables, this becomes extremely difficult and results are hard to interpret. To deal with these problems, we develop the 'block principal component analysis' method to be discussed in the following sections.

3. BLOCK PRINCIPAL COMPONENT ANALYSIS

Ordinary principal component analysis needs to find an orthogonal matrix $\mathbf{W}$ such that $\mathbf{W}'\Sigma\mathbf{W}$ is diagonal. In a very extreme case when all of the components of $\mathbf{X}$ are independent, the p principal components are the p components of $\mathbf{X}$, and $\mathbf{W}$ is merely some permutation of the identity matrix, rearranging the components of $\mathbf{X}$ according to their variances. If the random vector $\mathbf{X}$ can be partitioned into k uncorrelated random subvectors, so that $\mathbf{\Sigma}$ has diagonal blocks, then performing principal component analysis with $\mathbf{X}$ is equivalent to performing principal component analysis with each subvector and then combining all the principal components

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from all subvectors. This simple fact leads to the consideration of 'block principal component analysis' even when $\mathbf{X}$ does not have uncorrelated partitions.

Let $\mathbf{X}$ be partitioned as $\mathbf{X}=(\mathbf{X}_1',\ldots,\mathbf{X}_k')'$ with $\mathbf{X}_i$ being p_i-dimensional, where $p_1+\cdots+p_k=p$, and $\boldsymbol{\Sigma}$ be partitioned accordingly as

$$\boldsymbol{\Sigma}=\begin{pmatrix}\boldsymbol{\Sigma}_{11} & \boldsymbol{\Sigma}_{12} & \cdots & \boldsymbol{\Sigma}_{1k}\\ \cdot & \cdot & \cdots & \cdot\\ \cdot & \cdot & \cdots & \cdot\\ \boldsymbol{\Sigma}_{k1} & \boldsymbol{\Sigma}_{k2} & \cdots & \boldsymbol{\Sigma}_{kk}\end{pmatrix} \tag{2}$$

Let $\mathbf{W}_i=(\mathbf{w}_{i1},\ldots,\mathbf{w}_{ip_i})$, $i=1,\ldots,k$, be an $p_i\times p_i$ orthogonal matrix such that

$$\mathbf{W}_i'\boldsymbol{\Sigma}_{ii}\mathbf{W}_i=\boldsymbol{\Lambda}_i=\operatorname{diag}(\lambda_{i1},\ldots,\lambda_{ip_i}),\quad \lambda_{i1}\geqslant\cdots\geqslant\lambda_{ip_i} \tag{3}$$

so that $\mathbf{w}_{ij}$, $j=1,\ldots,p_i$, is an eigenvector of $\boldsymbol{\Sigma}_{ii}$ corresponding to the eigenvalue λ_{ij}. Put $\mathbf{U}_i=\mathbf{W}_i'\mathbf{X}_i=(U_{i1},\ldots,U_{ip_i})'$, then the p_i components U_{ij}, $j=1,\ldots,p_i$, of $\mathbf{U}_i$ define the p_i principal components, referred to as 'block' principal components, with respect to the random vector $\mathbf{X}_i$, the ith block of variables of $\mathbf{X}$.

Now define

$$\mathbf{Q}=\operatorname{diag}(\mathbf{W}_1,\ldots,\mathbf{W}_k) \tag{4}$$

also an orthogonal matrix, and

$$\mathbf{Y}=\mathbf{Q}'\mathbf{X}=(\mathbf{U}_1',\ldots,\mathbf{U}_k')' \tag{5}$$

a random vector combining all 'block' principal components, then

$$\operatorname{cov}(Y)=\boldsymbol{\Omega}=\mathbf{Q}'\boldsymbol{\Sigma}\mathbf{Q}=\begin{pmatrix}\boldsymbol{\Lambda}_1 & \mathbf{W}_1'\boldsymbol{\Sigma}_{12}\mathbf{W}_2 & \cdots & \mathbf{W}_1'\boldsymbol{\Sigma}_{1k}\mathbf{W}_k\\ \cdot & \cdot & \cdots & \cdot\\ \cdot & \cdot & \cdots & \cdot\\ \mathbf{W}_k'\boldsymbol{\Sigma}_{k1}\mathbf{W}_1 & \mathbf{W}_k'\boldsymbol{\Sigma}_{k2}\mathbf{W}_2 & \cdots & \boldsymbol{\Lambda}_k\end{pmatrix} \tag{6}$$

Note that $\boldsymbol{\Omega}$ and $\boldsymbol{\Sigma}$ have the same eigenvalues, and in particular, $\operatorname{tr}(\boldsymbol{\Omega})=\operatorname{tr}(\boldsymbol{\Sigma})$, where tr stands for the trace (sum of all diagonal elements) of a matrix. Hence $\mathbf{X}$ and $\mathbf{Y}$ have equal total variance. Let $\mathbf{W}$ be defined as in (1), and

$$\mathbf{R}=\mathbf{Q}'\mathbf{W} \tag{7}$$

then $\mathbf{R}$ is also an orthogonal matrix and

$$\mathbf{R}'\operatorname{cov}(Y)\mathbf{R}=\mathbf{W}'\boldsymbol{\Sigma}\mathbf{W}=\operatorname{diag}(\lambda_1,\ldots,\lambda_p) \tag{8}$$

that is, the p principal components of $\mathbf{Y}$ are identical to those of $\mathbf{X}$.

Hence, we can obtain the principal components of a random vector $\mathbf{X}$ by two steps. In the first step, we group the variables in $\mathbf{X}$ into several blocks, and then derive principal components for each block of variables. In the second step, we define a new random vector $\mathbf{Y}$ by combining all the 'block' principal components and then obtain the principal components of $\mathbf{Y}$, which are identical to the principal components of $\mathbf{X}$.

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The geometrical interpretation of block principal component analysis is quite clear. The p-dimensional random vector $\mathbf{X}$ represents the p axes in a p-dimensional space. The p principal components rotate the $\mathbf{X}$-space to one whose axes are defined by the p principal components. In order to rotate the original space to its desired direction, we can first group the axis and rotate the axis within each group and then do an overall rotation to achieve the desired direction.

From the mathematical derivation above, we notice that this procedure always yields the principal components of $\mathbf{X}$, regardless of how the blocks are defined. The choice of blocks, however, does have effects on several aspects. First, if the $\mathbf{X}$ can be divided into uncorrelated blocks, then the components in $\mathbf{Y}$ are the principal components of $\mathbf{X}$, and there is no need to orthogonalize $\mathbf{Y}$. Second, even when $\mathbf{X}$ cannot be partitioned into uncorrelated blocks, if the off-diagonal terms $\mathbf{W}_i'\mathbf{\Sigma}_{ij}\mathbf{W}_j$ are relatively small, as measured, say, by a matrix norm (for example, squared sum of squares of all elements), then without losing much information, we can still use the components of $\mathbf{Y}$ as approximation to the principal components of $\mathbf{X}$. Third, when reduction in dimension and in the number of variables is conducted within each block, which will be discussed in the next section, we would expect that variables within each block are much more correlated than variables from two different blocks, so that selection of dimensions and of variables from one block will not be much affected by selection of variables from another block. For these reasons, we recommend grouping the variables into blocks according to their correlation. This can be achieved by clustering the variables using a proper function of Pearson's correlation coefficient as the measure of similarity between variables; one such measure is given in Section 5 of the paper.

4. DIMENSION REDUCTION AND VARIABLE SELECTION

4.1. Dimension reduction

A major application of principal component analysis is to reduce data dimension so that the data structures can be explored or even visualized in a low-dimensional space. When data dimension is extremely high, block principal component analysis allows us to reduce data dimension more effectively without losing much information. We propose the following procedure to achieve low dimension. Suppose k blocks, $\mathbf{X}_i$, with dimension p_i and covariance matrix $\mathbf{\Sigma}_{ii}$, $i=1,\ldots,k$, of the original variables $\mathbf{X}$, are determined according to the correlation between variables. For each block $\mathbf{X}_i$ we derive the p_i principal components, and retain only the first q_i ($< p_i$) principal components, say, U_{ij}, $j=1,\ldots,q_i$, so that the total variance explained by these q_i principal components is $\pi_i \operatorname{tr}(\mathbf{\Sigma}_{ii})$, where $0<\pi_i<1$. Now define

$$\tilde{\mathbf{Y}}=(U_{11},\ldots,U_{1q_1},\ldots,U_{k1},\ldots,U_{kq_k})' \tag{9}$$

a variable combining all principal components selected from each block. We then obtain the principal components of $\tilde{\mathbf{Y}}$, and choose the first f principal components, say $Z_1,\ldots,Z_f$, which explain a high percentage of $100\tilde{\pi}$ per cent (for example, $\tilde{\pi}=95$ per cent) of the total variance of $\tilde{\mathbf{Y}}$. Data visualization and classification with the original variable $\mathbf{X}$ is then conducted based on these f principal components.

These block principal components preserve many optimal properties of the ordinary principal components: (i) $Z_1,\ldots,Z_f$ are uncorrelated; and (ii) $\operatorname{var}(Z_1)\geqslant\cdots\geqslant\operatorname{var}(Z_f)$. However, these variances are no longer the eigenvalues of $\mathbf{\Sigma}$, the covariance matrix of the original

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variables $\mathbf{X}$. Instead, they are the eigenvalues of the covariance matrix of $\tilde{\mathbf{Y}}$; (iii) the total variance of $Z_1,\ldots,Z_f$ is

$$\tilde{\pi}\,\mathrm{tr}[\mathrm{cov}(\tilde{\mathbf{Y}})]=\tilde{\pi}\sum_{i=1}^{k}\left[\sum_{j=1}^{q_i}\mathrm{var}(U_{ij})\right]=\tilde{\pi}\sum_{i=1}^{k}\pi_i\,\mathrm{tr}(\boldsymbol{\Sigma}_{ii})$$

which accounts for 100π per cent of the total variance of $\mathbf{X}$, where

$$\pi=\frac{\tilde{\pi}\sum_{i=1}^{k}\pi_i\,\mathrm{tr}(\boldsymbol{\Sigma}_{ii})}{\mathrm{tr}(\boldsymbol{\Sigma})}=\frac{\tilde{\pi}\sum_{i=1}^{k}\pi_i\,\mathrm{tr}(\boldsymbol{\Sigma}_{ii})}{\sum_{i=1}^{k}\mathrm{tr}(\boldsymbol{\Sigma}_{ii})}$$

We hence have

$$\pi\geqslant\tilde{\pi}\,\min\{\pi_i\} \tag{10}$$

When using principal components to explore (for example, cluster, visualize) the data, we expect that the leading components explain most of the variance so that they will reveal the true nature of the data structure; (10) asserts that block principal components $Z_1,\ldots,Z_f$ will retain most of the variance if, within each block and for the final principal component analysis, the selected principal components explain most of the variance. For example, if $\pi_i\geqslant 95$ per cent, $i=1,\ldots,k$ and $\tilde{\pi}>95$ per cent, then $\pi>90$ per cent.

4.2. *Variable selection*

When the number p of variables is very large, many variables can be highly correlated with each other and some may become redundant when the rest are being used to explore date structure. For example, in a gene microarray experiment where gene expression of a large number of genes is obtained for a number of tissues, tissue classification based on all genes may be quite similar to that based on a small group of genes. If this is the case, then with respect to tissue classification, only these genes are informative and the rest become redundant, assuming that using all the genes indeed captures the real structure of the data. It is therefore important to select variables that contain almost all information, with respect to certain statistical properties, that all variables would contain.

Block principal component analysis can be used to select these variables. We propose the following two steps:

Step 1. Divide the original variable $\mathbf{X}$ into k blocks, $\mathbf{X}_i$, $i=1,\ldots,k$, according to correlation between variables.

Step 2. For each block $\mathbf{X}_i$, conduct principal component analysis and select the first q_i leading principal components such that the total variance of $\mathbf{X}_i$ is explained by a satisfactory amount, say, at least 95 per cent. Examine the coefficients (or loadings in many principal component analysis literatures) of the variables in $\mathbf{X}_i$ in these q_i leading components and retain only those variables with large coefficients. Combine all the variables selected from each block and then use only these variables for further analysis.

A third step may also be useful if the number of variables selected is still too large:

Step 3. Conduct principal component analysis again, but based only on the variables selected in step 2. Select the first several leading principal components to explain most of the

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variance. Then examine the variables again and retain those with large coefficients in these leading combinations.

There is no universal criterion for how many and which variables should be selected from the leading principal components. Jolliffe [1] recommended choosing a variable from each leading principal component with the largest absolute coefficient, if the variable has not been selected from previous leading components. In practice some modifications of Jolliffe's procedure may also be effective. For example, one can choose several variables from each leading principal component with the largest absolute coefficients. For more discussion, see reference [1].

In the next section, we demonstrate the block principal component analysis method using the well-known NCI60 human cancer cell-line data [7] to select a group of genes to visualize/cluster the cell lines. The result shows such selection to be quite effective.

5. APPLICATION TO GENE MICROARRAY ANALYSIS: AN EXAMPLE

The NCI60 database contains expression of more than 9000 genes of 60 human cancer cell lines from nine types of cancer including colorectal, renal, ovarian, breast, prostate, lung and central nervous system, as well as leukaemia and melanomas. Gene expression levels are expressed as $-\log(\text{ratio})$, where ratio = the red/green fluorescence ratio after computational balancing of the two channels. Readers are referred to reference [7] for more details. The data have been made public for analysis on the authors' web site http://discover.nci.nih.gov. To get familiar with the DNA microarray technology, readers are referred to references [5] and [6] for more information.

One of the objectives of this study is to explore the relationship between gene profiles and cancer phenotypes. Scherf *et al.* [7] used a clustering analysis method to study the relationship. They provide the clustering tree of the 60 cell lines, based on 1376 genes, and showed that most of the cell lines cluster together according to their phenotypes (see Figure 2a of reference [7].) One important question is whether a smaller group of genes can preserve the same relationship structure.

We use a selection method based on block principal component analysis, as described in Section 4, to tackle this issue. For simplicity, we study only cell lines from three types of cancer, colorectal (7 cell lines), leukaemia (6 cell lines) and renal (8 cell lines); each cell line has microarray expression of the same 1416 genes. The data set of interest, 21 cell lines (being the subjects) and 1416 genes (being the variables), hence form a 21×1416 matrix, representing 21 data points (21 rows of the matrix) in a 1416-dimensional data space. The complete-linkage clustering tree based on these 1416 genes is shown in Figure 1(a). The dendrogram is consistent with that in reference [7], and shows clearly that the 21 cell lines cluster according to their cancer phenotypes. The readers are reminded that phenotype information is not used in the clustering, but only to validate the clustering results. One renal cell line marked as 'RE8', which is farther from the rest of renal cell lines, has been recognized to have some special feature (see reference [7] for detail).

We now seek to determine the blocks for the 1416 genes. Figure 2 shows a plot of semi-partial R^2 versus the number of clusters using complete-linkage algorithm and $d_{ij} = \arccos(|\rho_{ij}|)$ as a measure of dissimilarity between gene i and gene j, where ρ_{ij} is the Pearson correlation

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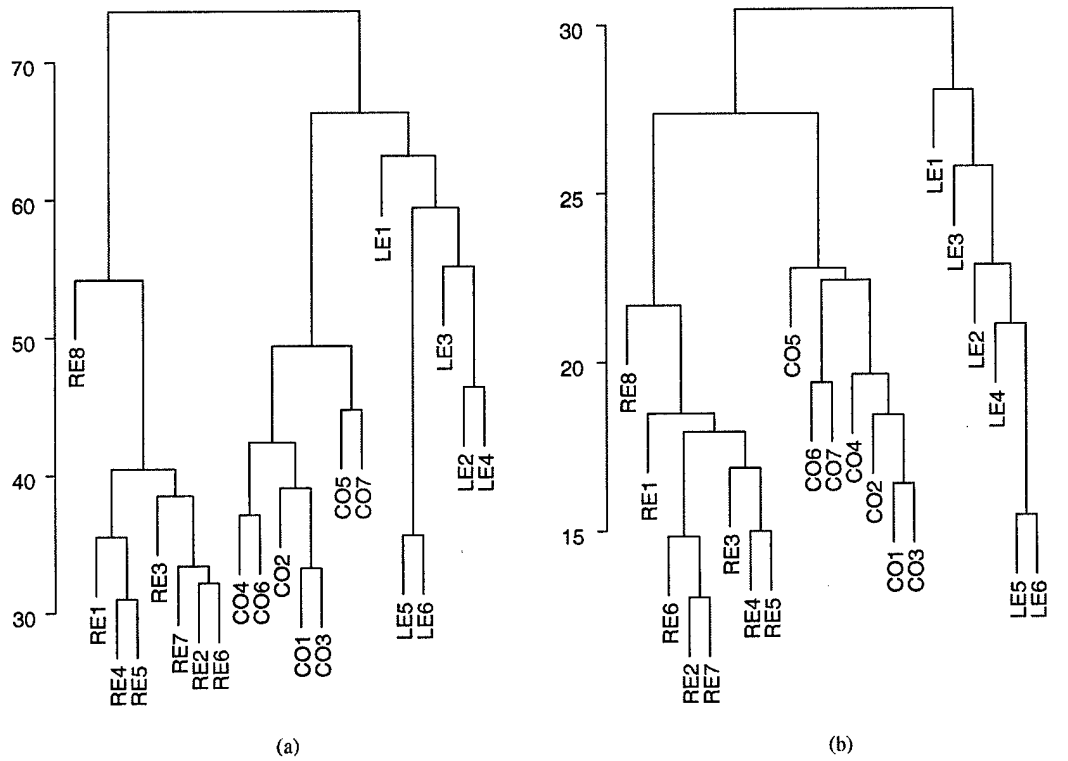

Figure 1. Dendrogram of complete linkage hierarchical clustering of 21 cell lines: (*a*) tree based on 1416 genes; (*b*) tree based on 200 genes. CO is colorectal, LE is leukaemia and RE is renal.

coefficient. The semi-partial R^2 measures the loss of homogeneity when two clusters are merged. Define SS_T as the corrected total sum of squares of all subjects and summed over all variables. For a certain cluster C, let SS_C be the corrected total sum of squares of all subjects in cluster C summed over all variables. Then the semi-partial R^2 for combining two clusters C_1 and C_2 into one cluster C is $(SS_C - SS_{C_1} - SS_{C_2})/SS_T$. A large semi-partial R^2 indicates significant decrease in homogeneity. Since subjects within the same cluster should be similar, two clusters should not be combined as one cluster if the semi-partial R^2 is large. In practice we determine the number of clusters by minimizing the semi-partial R^2; a plot of the semi-partial R^2 versus the number of clusters is extremely helpful. More discussion and computation of semi-partial R^2 can be found in reference [8]. Other statistics can also be used to determine the number of clusters in the data. Milligan and Cooper [9] examined 30 procedures for determining the number of clusters, including several variations based on sum of squares.

For the cancer cell-line microarray data, the semi-partial R^2 becomes nearly flat after 14 clusters. This indicates that the 1416 genes can be approximately divided into 14 clusters; further dividing the data gains little in reducing heterogeneity. These clusters of genes determine the blocks within each of which principal component analysis will be conducted. The number of genes in the blocks ranges from 43 to 158 (Table I). Principal component analysis

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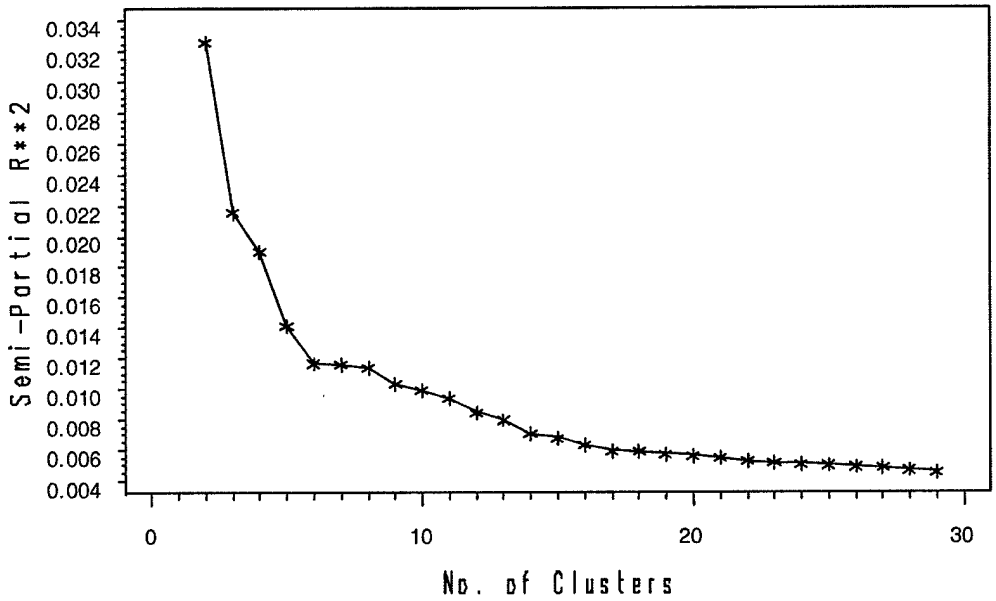

Figure 2. Determining number of blocks: plot of semi-partial R^2.

Table I. Summary of 14 gene blocks.

	Block													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Number of genes	107	154	88	158	68	152	84	136	44	84	143	82	73	43
Number of PCs	14	13	15	15	14	15	14	15	14	16	16	14	14	11
Per cent variance	95.6	95.3	95.2	95.3	95.2	96	95.1	95.2	96	96	95.1	96	95.6	95.6

is conducted within each block, and the first several leading principal components are then selected, resulting in a total of 200 principal components. For each block, selected principal components explain >95 per cent of total variance in that block. For each block, Table I lists the number of genes, the number of selected principal components and the percentage of total variance explained by these leading components.

For each block, genes with largest coefficients in the selected leading principal components are retained, using Jollife's one variable per leading component method. This yields a total of 200 genes for further analysis.

The first three leading principal components, computed based on the 1416 genes, explain only 49 per cent of the total variance. Two- or three-dimensional visualization of the data based on these principal components can be very misleading. We validate these selected 200 genes by deriving a hierarchical clustering tree for the 21 cell lines based on gene expressions. The dendrogram is shown in Figure 1(b). It is remarkably similar to the one based on all 1416 genes (Figure 1(a)). Both illustrate that cell lines with the same phenotype are more similar than those from different phenotypes. This shows that a much smaller number of genes

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can provide the same insight for the data as the whole set of genes, and block principal component analysis provides an effective way to achieve this. Note that a hierarchical clustering dendrogram, obtained based on a set of variables, is essentially the same as the hierarchical clustering dendrogram obtained based on leading principal components, provided that these leading components explain most of the variation among the variables. The remarkable resemblance between Figure 1(*a*) and Figure 1(*b*) further demonstrates the effectiveness of the block principal component analysis method, as compared to the ordinary principal component analysis.

6. DISCUSSION

In this paper we show that a much smaller number of genes can provide the same insight for the cancer phenotypes as the whole set of genes. We demonstrate that block principal component analysis is an effective way to select these genes. This kind of analysis is 'unsupervised', a term popular in neural network/pattern recognition [10]; cancer phenotypes are used only to validate the algorithm and analysis.

Selection of informative genes in the microarray setting, and other settings as well, is by no means an easy task, especially when the analysis is unsupervised. Very likely the choices of genes are not unique; there might exist several groups of genes that provide the same classification. Biostatisticians should provide every potential group of genes to the medical investigators and hopefully a meaningful group of genes can be determined by combining the statistical guidance and biological knowledge. Indeed, some preliminary selection of genes based on biological knowledge is extremely valuable, even before any statistical analysis is conducted. It should be noted, however, that genes that are biologically similar/dissimilar may not be statistically similar (correlated)/dissimilar (uncorrelated).

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